Characterizing putative cellular mediators of West Nile virus infections in bird and mosquito tissues

BY

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Abstract

West Nile virus (WNV) is a mosquito-borne virus that infects many bird species. Examination of American crows and house sparrows from the Winnipeg region confirmed that WNV levels were at least 1000 times higher in crows than sparrows. No species differences were observed in the level of transcripts encoding a putative WNV receptor, β3 integrin. Differences in mosquito vector competence can be due to differences in the ability of WNV to enter mosquito cells. Using RNAi techniques, the role of two clathrin coat adaptor proteins in facilitating WNV infections in mosquito cells was examined, and the findings suggest that these proteins may act as resistance factors in *Aedes aegypti*, and as susceptibility factors in *Culex quinquefasciatus*. These findings will contribute to our understanding of the molecular basis of vector competence in different mosquitoes, and may help us determine whether other species could serve as potential vectors of this health-threatening virus.

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Chapter 1: General Introduction

1.1 West Nile Virus Epidemiology

West Nile virus (WNV) is a mosquito-borne arbovirus of the genus *Flavivirus*, a genus that includes over 100 viruses such as Dengue virus, Yellow Fever virus, and Japanese encephalitis virus. It was first documented in 1937 in Uganda and gradually spread throughout Africa, Asia, Europe, and the Middle East (World Health Organization 2011), but was only introduced to North America in 1999. Initially, several crows and exotic birds were found dead in New York City which, at the time, was attributed to an unknown cause. Subsequently, citizens of NYC began reporting flu-like illnesses and antibody screening determined that they, along with the dead birds, were infected with WNV (CDC 1999). Following the initial outbreak, the virus rapidly spread across the USA and into many parts of Canada as well as Central and South America, establishing a wide range in the western hemisphere (Beasley 2005). This spread has been mediated by the large number of host species it infects, including many migratory birds, and numerous virus-transmitting mosquitoes (Rappole *et al.* 2000). It is now endemic in Africa, Asia, Australia, Europe, the Middle East, and North America and has become the most common cause of arboviral encephalitis worldwide (Chancey *et al.* 2015). Symptoms are present in approximately 20% of infected people and typically present as flu-like illness; however serious neuroinvasive conditions, including encephalitis and meningitis, can occur in approximately 5% of infected individuals; some of which are fatal (Beckham & Tyler 2009; Kramer & Bernard 2001). To date, human cases in the US total 41,679, of which 18,746 presented as neuroinvasive disease and 1,753 were fatal (CDC 2015). In Canada, the first reports of WNV infections in humans began in 2002 and currently, of the approximately 5,200 total documented cases, 53 have been fatal (IPAC Canada 2014; Public Health Agency 2015). Differences in surveillance of

infected individuals and the availability of public health records may, however, underestimate these numbers because of the high frequency of asymptomatic cases. Over the past ten years, the incidence of WNV has declined; however in the past two years (2012-2014), there has been a drastic increase in the number of WNV cases in the USA which are comparable to the numbers of cases during the largest outbreaks seen in 2002 and 2003 (CDC 2015). Annual fluctuations in climate can affect mosquito and bird populations (Ostfeld 2009; Harvell *et al.* 2002), and as incidences of WNV have been associated with high mosquito activity (Nielsen 2008), the number of people contracting the virus in any given year is variable, and the occurrence of future outbreaks is extremely likely.

1.2 WNV Transmission Cycle

Mosquitoes facilitate WNV transmission and birds are the natural vertebrate hosts that act to maintain the virus cycle (Artsob *et al.* 2009; Beasley 2005). Certain mosquito species, such as those in the genus *Culex*, act as bridge vectors that transmit the virus between infected birds and other vertebrate hosts including humans, other species of mammals, reptiles, and anurans (Artsob *et al.* 2009; Kilpatrick *et al.* 2005; Turell *et al.* 2005). Although transmission through animal-to-human or human-to-human contact has not been documented to date, infections have occurred through organ transplant, blood transfusions, intrauterine transmission, and breast milk, with one reported case of trans-placental transmission (World Health Organization 2011). Curiously, in some bird species such as corvids (e.g. crows, ravens, jays), the virus quickly replicates to high titers and results in high mortality, while in others, rapid replication does not seriously affect lifespan (Del Amo *et al.* 2014; Reisen *et al.* 2006; Komar 2003). Similarly, the virus infects mosquito species differently, with some species, such as those of the genus *Culex*, having high

WNV infection rates, while those of the genus *Aedes* show considerably lower infection rates (Brault 2009; Turell *et al.* 2005; Turell *et al.* 2000). An important factor that affects mosquito vector competence (i.e. a vector's capacity to acquire and transmit microbial pathogens) is the insect's susceptibility to infection by the pathogen, but to date, these factors have not yet been identified. Additionally, the ability of the virus to cause disease and/or mortality in a host organism may be related to host susceptibility to the virus, the amount of virus found in the host organism, and the location of the virus within the host organism. In both birds and mosquitoes, the factors that might limit or exacerbate WNV infection in different species have not been adequately determined.

1.3 The Role of Mosquitoes in WNV Transmission

The replication cycle of WNV within mosquitoes begins when the virus enters the gut after an infected blood meal is taken by an adult female. While the meal is digested, virions enter the epithelial cells of the midgut where they replicate (Vaidyanathan & Scott 2006). Mature virions cross through the epithelial cells, enter the hemocoel, and migrate into tissues such as the salivary glands ((Figure 1.1; Vaidyanathan & Scott 2006). To date, no studies have reported WNV entering the crop, presumably because it functions in the storage of sugar meals; however because the virus is able to migrate to salivary gland tissue, it is possible that it is also able to penetrate crop tissue. Viral infection in mosquitoes typically requires an incubation period of approximately 5-14 days before it can be transmitted to another host and since the lifespan of certain mosquito species ranges from one week to several months, one infected adult female can transmit the virus to multiple hosts (Turell *et al.* 2000). During subsequent feedings, the mosquito transmits the virus to the next host through its saliva. Vertical transmission between

females and embryos has been documented in many species and has facilitated overwintering of the virus in harsh climates (Nelms *et al.* 2013; Baqar *et al.* 1993).

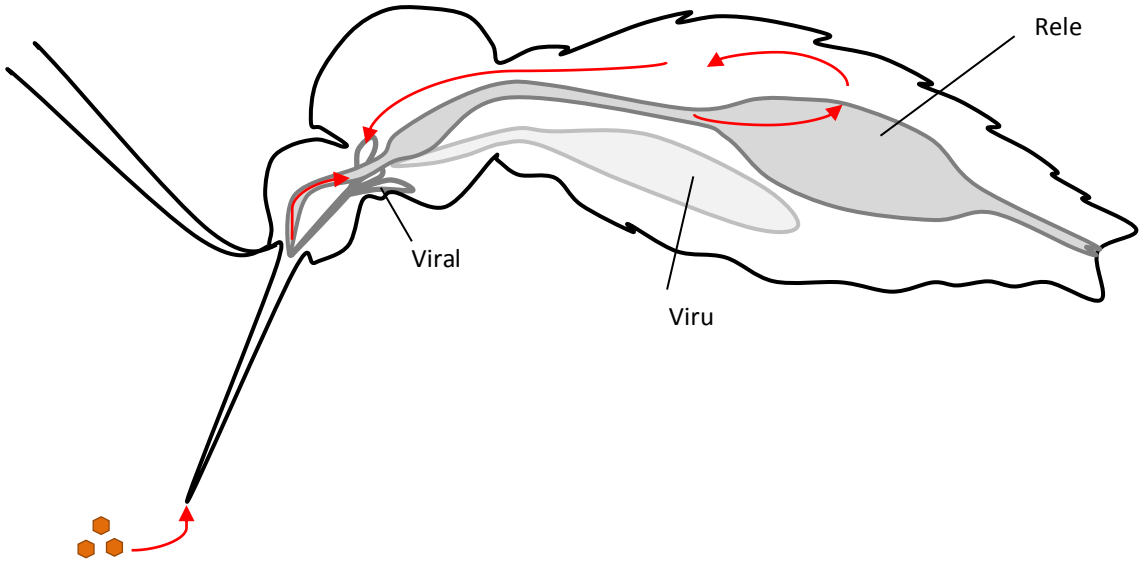


Figure 1.1. Internal mosquito anatomy highlighting the pathway through the tissues that WNV takes as part of the infective cycle. **SG** – salivary glands; **CR** – crop; **MG** – midgut. The path of infection is indicated by the red arrows.

Culex spp. mosquitoes are the primary vectors of WNV in North America (Beasley 2005) although to date, 65 different mosquito species have been identified as having been infected with WNV, including *Aedes spp., Anopheles spp., and Culiseta spp.* (CDC 2012). Some species, especially many of the *Culex spp.*, are considered more serious vectors than others because they are not strictly ornithophilic and can feed on humans and other animals besides birds (Molaei *et al.* 2006). They are also more susceptible to developing high viremia from bird hosts (Turell *et al.* 2000). The most competent *Culex spp.* enzootic and bridge vectors are *Cx. pipiens, Cx. quinquefasciatus, Cx. restuans, Cx. salinarius, and Cx. tarsalis* (Kilpatrick *et al.* 2005; Turell *et al.* 2005). Alternately, *Aedes spp*. are not considered a primary vector largely because they tend to feed only on mammals and not on birds (Turell *et al.* 2005), thus reducing the risk of ingesting a blood meal containing WNV. It is unclear why WNV is able to replicate to high numbers in

certain mosquito species whereas in others, levels of the virus remain relatively low, although it has been proposed that intrinsic barriers within the mesenteron and salivary glands limit viral infection in certain mosquito species (Kramer *et al.* 1981). Different mosquito species have different distributions across North America and globally (Beasley 2005), and therefore the most important vector in different regions will vary, as each species' location is dependent on preferences in the ecology and climate of a particular area (Artsob *et al.* 2009). Environmental conditions relating to temperature and water availability can have an effect on mosquito populations and therefore the frequency of mosquito-borne virus infection, and consequently, the WNV transmission cycle (Brinton 2002). *Culex tarsalis* is the primary vector in the Western United States and Canada, whereas in Eastern parts of North America, members of the *Culex pipiens* complex, including *C. quinquefasciatus*, are the predominant species involved in the WNV transmission cycle (Kilpatrick *et al.* 2006; Kilpatrick *et al.* 2005).

Although mosquitoes become infected with WNV and the virus is able to replicate within these vectors, they do not show signs of illness and there are no reports of WNV-mediated death in these organisms. This is likely due to the presence of certain chitins, lectins, carbohydrates, and other proteins in the midgut which prevent pathogen attachment and entry into epithelial cells (Colpitts *et al.* 2012; Shao *et al.* 2001). Additionally, increased apoptosis in infected epithelial cells of the midgut can limit viral dissemination (Vaidyanthan & Scott 2006). There is also evidence of an immune response towards the virus through the RNA interference (RNAi) and innate immune pathways, including Toll, JAK-STAT, and Immune Deficiency (IMD) (Hillyer 2010). By identifying key factors that increase vector competence, improved efforts can be made to control certain mosquito species, especially in areas where certain diseases are endemic, to limit the spread and severity of arboviral disease.

1.4 The Role of Birds in WNV Transmission

Birds play an important role in public health because they can become infected by many different pathogens, some of which are transmissible to humans. The migration patterns of certain bird species in particular can result in the long-range spread of pathogenic organisms, which creates the potential for the establishment of new endemic regions of disease along migration routes (Reed *et al.* 2003; Rappole *et al.* 2000). How WNV first arrived in North America is still a matter of speculation; while some people suggest that WNV was introduced by an infected migratory bird, others suggest that an infected exotic bird was imported from an overseas zoo (Johnston & Conly 2000). Wild birds are the natural hosts of WNV, and to date, 332 bird species in Canada and the USA have been positively identified as being infected with WNV (CDC 2014; CCWHC 2014). Although the virus is able to infect and replicate within many bird species, curiously, in some species, such as corvids (crows, ravens, magpies, etc.), it can quickly replicate to high titers, resulting in high mortality rates in these species (Reisen *et al.* 2006; Brault *et al.* 2004) whereas in contrast, WNV replicates quickly in members of the Passeridae family (sparrows, finches, etc.) but seldom do these birds display serious consequences of the infections (Langevin *et al.* 2005; Reisen *et al.* 2005). Interestingly, birds in Europe, Africa, Asia and the Middle East have low mortality when infected whereas in the Americas, the virus can be highly pathogenic and cause high death rates in certain species such as corvids (World Health Organization 2011). Most bird species show few symptoms once they become infected, and become viremic several days after their initial exposure (Peterson & Roehrig 2001). The virus can remain in the birds for quite some time; in ducks and pigeons, for example, the virus can persist for 20 to 100 days following inoculation with WNV (Johnston & Conly 2000). In contrast, corvids develop severe illness and have a high mortality rate. For this

reason, corvids have been used as sentinels for WNV in new endemic areas, and are currently being used in surveillance programs to monitor WNV outbreaks in regions where they have been known to occur (CCWHC 2014; Komar 2001). It is currently unknown why certain species develop severe symptoms including potentially fatal encephalitis (Guarner *et al.* 2004) while others remain asymptomatic.

1.5 WNV Structure

WNV is a spherical, lipid-enveloped flavivirus containing a single stranded, positive sense RNA genome, meaning the viral RNA can act directly as a template for translation (Mukhopadhyay *et al.* 2005; Brinton 2002). Three structural proteins, the capsid protein (C, initially expressed as anchored C, anC), membrane protein (M, initially expressed as a precursor to membrane, prM), and envelope protein (E), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are translated from the RNA genome and are co-and post-translationally cleaved from a single polypeptide by host proteases to give rise to individual proteins (Fig. 1.2; Zhang *et al.* 2003; Brinton 2002). The NS1, NS2B, NS3, NS4A, and NS5 proteins have enzymatic functions necessary for viral replication whereas the NS2A, NS2B, NS4A, and NS4B are small hydrophobic molecules with no known enzymatic functions, although they are believed to play a role in viral replication, assembly, and localization (Chancey *et al.* 2015). The NS proteins are also responsible for regulating cell signalling and immune responses. The nucleocapsid core of the virus is comprised of multiple copies of C protein that surround the RNA genome (Zhang *et al.* 2003). In the immature virion, the prM glycoprotein forms a heterodimer with the E glycoprotein which is embedded in the lipid bilayer during viral assembly to prevent premature fusion to the host cell membrane and to ensure proper folding of

the E glycoprotein (Beasley 2005; Zhang *et al.* 2003). The pr peptide dissociates from the M protein at the time of mature virion assembly, and this conformational change allows the formation of E:E homodimers (Zhang *et al.* 2003). The E glycoprotein is comprised of 3 domains: a central structural domain (domain I), a dimerization domain that contains a fusion peptide (domain II), and an immunoglobulin (Ig)-like domain that has been implicated in receptor binding and fusion, and acts as the main target for neutralizing antibodies (domain III) (Mukhopadhyay *et al.* 2005; Zhang *et al.* 2003; Brinton 2002; Heinz & Allison 2000). Differential glycosylation occurs at the N-terminus of the E protein following passage in vertebrate or mosquito host cell, and these differences in glycosylation patterns of the E protein may relate to differences in neuroinvasiveness of the virus (Arjona *et al.* 2007; Beasley 2005). Additionally, when WNV is passaged in dipteran cells, the E glycoprotein is able to suppress immunity in murine systems by inhibiting cytokine production, but when it is passaged in mammalian cells, immune suppression does not occur (Arjona *et al.* 2007).

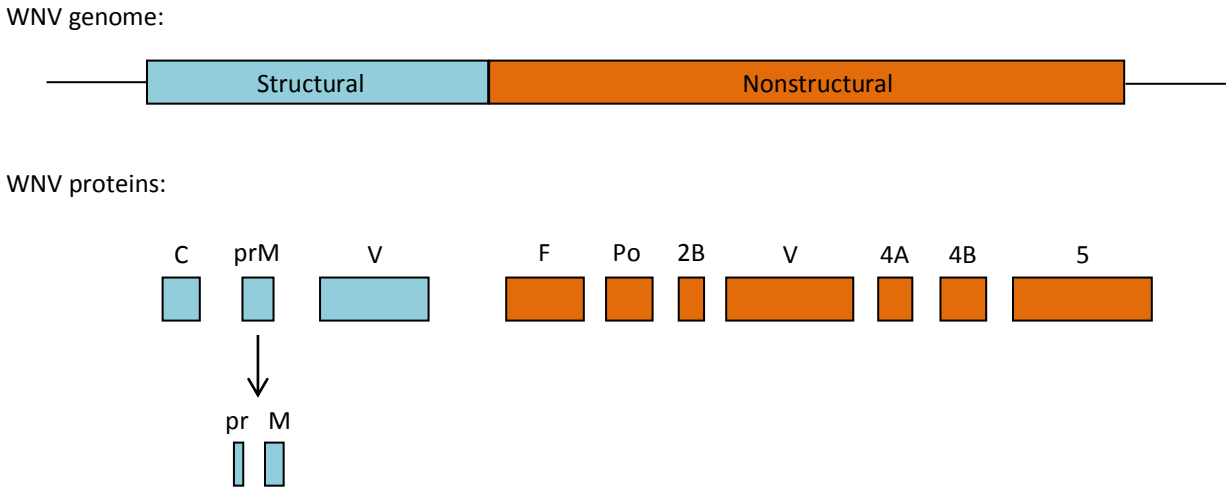


Figure 1.2. The RNA genome of WNV. The nonstructural gene region is shown in blue and the structural gene region is shown in orange. The single polyprotein is co- and post-translationally processed by viral and host cell proteases to form the mature viral proteins (shown below the genome). The genome and products are not drawn to scale.

Another factor affecting WNV pathogenesis is the genetic lineage of which it is a part. To date, phylogenetic analyses have divided WNV into five genetic lineages (Suthar *et al.* 2013), and these lineages can be further subdivided into strains and clades. For example, within lineage 1, clade 1a is found in North America, Europe, Asia, and the Middle East, and clade 1b (Kunjin virus) is primarily found in Australia (Bondre *et al.* 2007). Strains within lineage 1, such as NY99, are generally associated with high incidences of encephalitis and meningitis whereas strains within lineage 2 are typically less pathogenic and are primarily found within Africa (Bondre *et al.* 2007). Recently, however, isolates of the lineage 2 strain have been detected in Europe that were highly pathogenic in humans (Suthar *et al.* 2013; Berthet *et al.* 1997). Lineage 3 viruses have been isolated in Austria, and in Romaina and India, lineage 4 and 5 viruses have been detected respectively (Dinu *et al.* 2015; Bondre *et al.* 2007); however little is known about strains belonging to these lineages (Suthar *et al.* 2013; Bondre *et al.* 2007; Bakonyi *et al.* 2005; Lvov *et al.* 2004).

1.6 The WNV Infection Process

Flaviviruses enter the host through receptor-mediated endocytosis and the acidic environment found within the endosome changes the conformation of the E glycoprotein homodimer, allowing fusion between viral and cell membranes (Figure 1.3; Mukhopadhyay *et al.* 2005; Allison *et al.* 1995). This allows the release of the nucleocapsid into the host cell cytoplasm and subsequent dissociation of the capsid and RNA genome, which in turn initiates RNA replication and virus particle assembly (Brinton 2002). Within the lumen of the endoplasmic reticulum (ER), immature virion particles assemble and as they are processed through the trans-Golgi network, the pr peptide is cleaved from the M protein, making mature,

infectious virus particles (Elshuber *et al.* 2003). The mature virus acquires a lipid bilayer from the host cell as it is released through exocytosis, and the E and M proteins become integrated into this membrane (Mukhopadhyay *et al.* 2005).

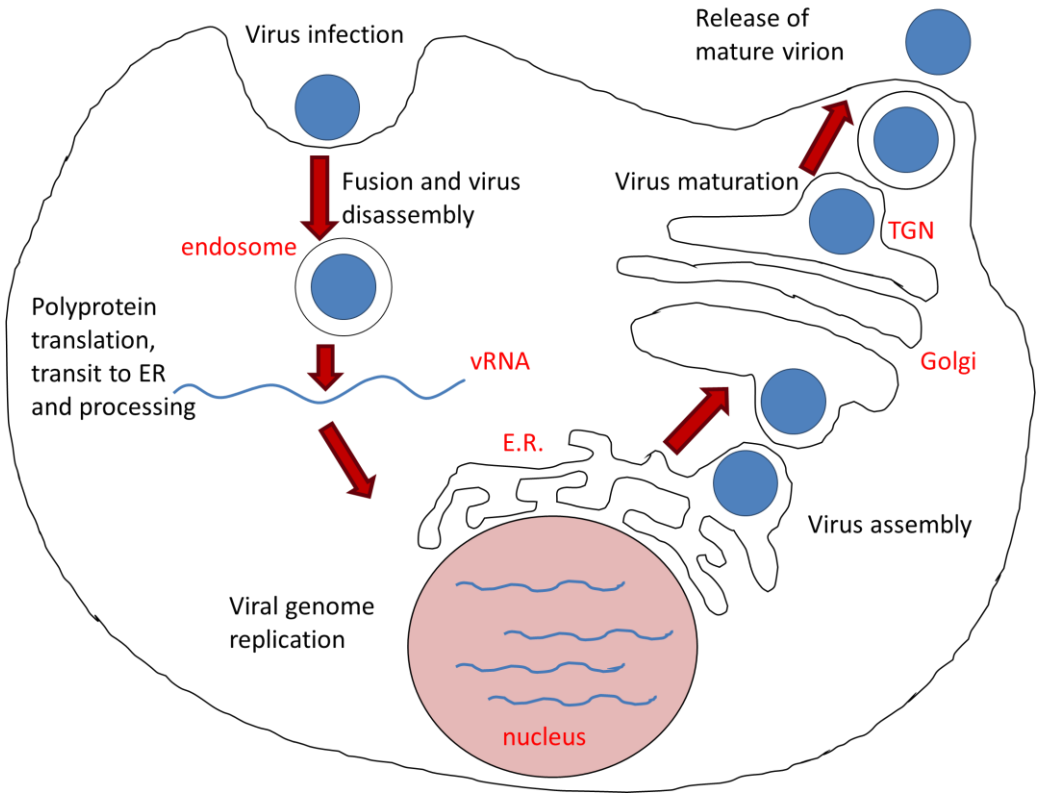


Figure 1.3. Flavivirus life cycle. Virions attach to the host cell surface and enter via receptor-mediated endocytosis. The acidic environment of the endosome induces conformational changes in the E protein that allows fusion and virus disassembly. The positive-sense RNA genome is translated into one polyprotein that becomes co- and post-translationally cleaved into individual proteins. On the surface of the ER, virus assembly takes place, which results in immature viral and subviral particles that are non-infectious. The immature particles are processed through the trans-Golgi network that induces the formation of mature, infectious virus particles. Mature virions and subviral particles are released by exocytosis.

The E glycoprotein has been implicated in the attachment of flaviviruses to host cells and it is widely accepted as the primary ligand of WNV (Arjona *et al.* 2007; Kanai *et al.* 2006; Heinz & Allison 2000). Specifically, the (Ig)-like domain III acts as the key binding site of the E

glycoprotein, although the fusion domain at the distal end of domain II has also been associated with viral attachment to host cells (Brinton 2009; Mukhopadhyay *et al.* 2005). The E glycoprotein is also the primary target of neutralizing antibodies when an immune response is mounted against WNV (Heinz & Allison 2000). Throughout the viral maturation process, the E glycoprotein undergoes several conformational changes. At a neutral pH, the E glycoprotein exists as a homodimer, but upon entering a cell, the acidic environment within the endosome allows its dissociation into monomers and subsequent re-association into homotrimeric 'spikes' (Beasley 2005; Mukhopadhyay *et al.*, 2005; Brinton 2002). These conformational changes allow the fusion of viral and host membranes by exposing the fusion peptide on domain II (Heinz & Allison 2000). Following membrane fusion, the virus is released into the cytoplasm of the host cell and is processed by host cell machinery to produce mature virions (Brinton 2001).

1.7 Putative Host Cell Receptors for WNV

Although the E glycoprotein serves as the primary ligand of WNV, host cell receptors for the virus have not been fully characterized and several candidate molecules have been proposed to act as receptor molecules in many different species (Colpitts *et al.* 2011; Cheng *et al.* 2010; Davis *et al.* 2006; Chu & Ng 2004). High homology exists among flaviviral E glycoproteins and it is possible that similar cell surface molecules bind a wide range of viruses within the *Flaviviridae* family (Anderson 2003). Many mosquito-borne flaviviruses including WNV possess an RGD (Arg-Gly-Asp) motif within domain III of the E glycopotein that mediates viral entry by binding to certain integrins in mammalian cells (Erb *et al.* 2010; Chu & Ng 2004; Ruoslahti 1996). However, under certain circumstances, the RGD peptide does not play a key role in WNV entry (Medigeshi *et al.* 2008) and therefore integrins may not be the primary

receptor for WNV in all organisms. Currently, several cell-surface molecules have been implicated in WNV attachment to host cells, including αVβ3 integrin in mammalian Vero cells (Chu & Ng 2004), and in human dendritic cells, the C-type lectin DC-SIGN has been identified as a putative receptor for WNV (Davis *et al.* 2006). In mosquitoes, cadherins and the C-type lectins mosGCTL and DC-SIGN are able to bind to the E glycoprotein of WNV to enable viral attachment to cells (Colpitts *et al.* 2011; Cheng *et al.* 2010). However, the roles of these molecules in the infection process have not been well defined, and nor have their roles in different species been explored. It is unclear whether these molecules act alone or in conjunction with other molecules to facilitate WNV attachment and fusion to host cells. Further, different tissues and different organisms may provide different receptors to the virus. It is possible that the virus is able to utilize multiple receptors during the infection process and there may be a range of different molecules on different cell types that act as receptors to WNV (Ren *et al.* 2007; Anderson 2003).

1.8 Thesis Objectives

There are several possible reasons why birds develop differential levels of infection from WNV including the number of receptors for the virus in key tissues, differences in innate and/or adaptive immunity, or differences in replication rates of the virus within cells. Similarly, in mosquitoes, differences in vector competence may be related to differential expression of certain genes in key tissues which affects WNV replication and transmission. The broad aim of my research project was to study WNV infection in two different animals involved in the cycling of the virus. Specifically, my aim was to examine the expression levels of genes putatively involved in WNV pathogenicity and transmission in different bird and mosquito tissues, and to determine

whether or not these genes play a role in WNV infection of host cells. Although several cell-surface molecules have been implicated in WNV attachment to mosquito (Cheng 2010; Colpitts *et al.* 2011) and vertebrate cells (Chu & Ng 2004), their roles in the infection process have not been well defined, nor have their roles in different species been explored. For this reason, I sought to examine whether certain cell surface molecules were expressed differently in bird host tissues such as the liver, heart, kidney, and brain, as these tissues are typically involved in the infection process. Similarly, in mosquitoes, I wanted to examine whether or not differences existed among expression levels of genes previously identified as playing a role in WNV infection among different mosquito species, and if differences in expression levels affected WNV infection of host cells.

By determining which receptors can facilitate WNV attachment to cells, we will better understand how this virus has adapted to so many different hosts in both vertebrates and invertebrates. If we can identify differential expression among certain key genes in mosquitoes that affect WNV infection and transmission, we may be able to explain some of the underlying aspects of mosquito-vector competence. Similarly, by identifying relevant receptors and their distribution in different tissues of birds, we may provide insights into different species' susceptibilities to this virus. This is important because it will give us clues to how we could control the impact of WNV infection by interrupting viral replication in mosquitoes or host species.

Chapter 2: Integrin and West Nile Virus Gene Expression in Bird Tissues

2.1 Introduction

Birds play a critical role in the WNV transmission cycle because they are the preferential hosts for certain species of mosquito, such as those of the *Culex* genus, which are considered the most effective vectors of the virus (Artsob *et al.* 2009; Brault 2009; Kilpatrick *et al.* 2005; Turell *et al.* 2005; Turell *et al.* 2000). WNV is transmitted to birds primarily through infected mosquito bites; however horizontal transmission through oral and cloacal contact as well as transmission via the consumption of infected prey or water have also been documented (Komar 2003; Banet-Noach *et al.* 2003; Nemeth *et al.* 2006; Swayne *et al.* 2001). Certain bird species are considered more important in the transmission cycle because the virus is able to replicate to high titres in the blood, therefore increasing the risk of infecting a mosquito vector when a blood meal is taken. Members of the *Corvidae* family, such as crows and jays, often become highly viremic; however, their role as hosts in the transmission cycle is controversial due to the rapid, high mortality associated with infection in these species (Komar 2003). Other species, such as those of the *Passeridae* family, which include sparrows and finches, typically have low mortality rates even with high viremia (Del Amo *et al.* 2014). Further, some species, such as chickens, develop an immunity to the virus, remain asymptomatic, and do not produce virus titers sufficient to infect mosquitoes (Langevin *et al.* 2001). These variations in the extent of viremia and mortality rates among host species and differences in host susceptibility to the virus could be attributed to differences in geographic range, body size, mating and breeding patterns, as well as co-evolution with the virus or other related viruses (Reisen *et al.* 2006; Figuerola *et al.* 2008; Reisen & Hahn 2007). Additionally, differential levels of infection from WNV could be influenced by the number of receptors for the virus in key tissues, differences in innate and/or adaptive immunity,

or differences in replication rates of the virus within cells.

Although the receptor for the E-glycoprotein of WNV is unknown for all tissues and all species, the αVβ3 integrin has been identified as a possible receptor for WNV in mammalian cells (Chu & Ng 2004), and it is possible that birds share a common, homologous protein that also acts as a receptor in these organisms. Additionally, differences in the amount of integrin in certain key bird tissues could influence WNV infection and account for the differences in pathology and mortality in different bird species. Integrins are a type of cell adhesion molecule from a family of integral membrane proteins that play some of the most important roles in making connections between cells and their extracellular matrix. They are cell surface glycoproteins that are composed of one alpha (α) and one beta (β) subunit, which together form non-covalently-linked heterodimeric pairs (Figure 2.1; Arnaout *et al.* 2005). Integrins possess a large extracellular domain that contains the N-terminus and many other protein domains, a single helical membrane-spanning region, and a short intracellular cytoplasmic tail, which contains the C-terminus (Srichai & Zent 2010). The cytoplasmic tail interacts with the cytoskeleton and intracellular signalling molecules. The extracellular component of the α subunit has up to 1104 residues, and at least nine known α subunits possess an additional ~190 amino acid sequence known as the von Willebrand factor A (vWFA) domain, as determined by crystallographic structure analyses (Lee *et al.* 1995). vWFA is a large glycoprotein domain found in a variety of plasma proteins, and in integrins, these regions are termed either αA or βA I-domains (Arnaout *et al.* 2005; Stoesser *et al.* 2000; Bronson & Fusi 1996). This domain has been involved in the formation of multiprotein complexes and interacts with a diverse array of ligands that mediate cell adhesion, migration, homing, pattern formation, and signal transduction (Stoesser *et al.* 2000). This domain also contains a metal ion-dependent adhesion site (MIDAS), a region that is

essential for binding of ligands and receptor function (Arnaout *et al.* 2005; Stoesser *et al.* 2000). Integrins that possess a vWFA domain in the β subunit (termed the βA domain) contribute to divalent cation-binding, particularly of Ca^{2+}, Mg^{2+} and Mn^{2+}, which allows for a strong ligand affinity to the β subunit, making the β subunit the main ligand binding site in these integrins (Niu & Chen 2011). The ligand-binding domain of all integrins is composed of the N-terminus of each subunit, which combines to form a globular peak (Aranout *et al.* 2005). This extracellular domain provides specificity to the receptor to which it binds, and specificity is further determined by the heterodimerization of the α- and β-subunits which occurs in the endoplasmic reticulum (Conesa *et al.* 2003).

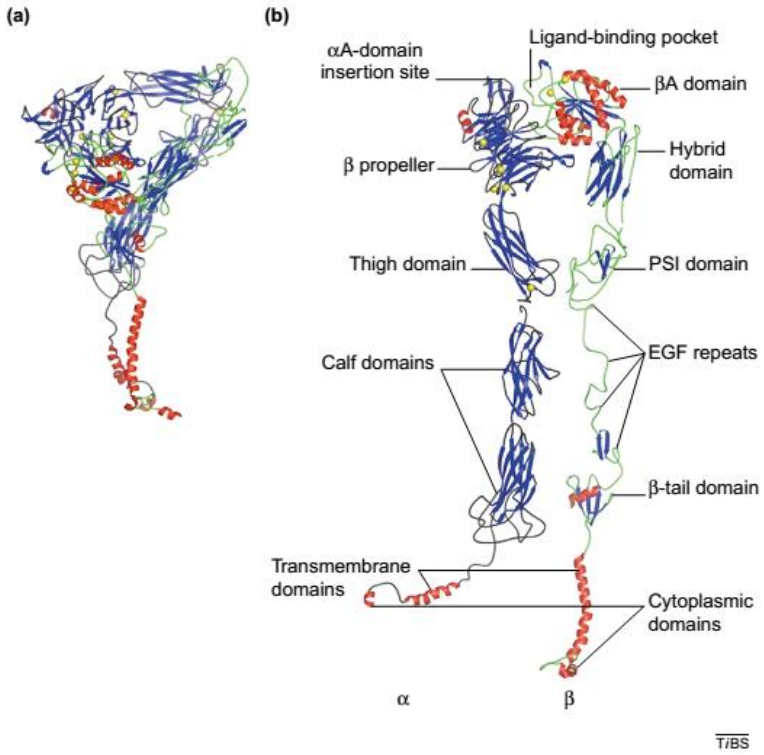


Figure 2.1. Structure of the αVβ3 integrin heterodimer. a) Integrin in the bent conformation. b) Integrin in the straightened conformation. (Reproduced with permission from Humphries *et al.* 2003. Permission granted July 16, 2015).

Integrins connect the cytoskeleton to the extracellular matrix and are able to transmit chemical signals into the cell (outside-in signalling) as well as to activate their ligand binding function through inside-out signalling (Reviewed in: Shen *et al.* 2012.; Harburger & Calderwood 2009). They are mainly localized in specialized structures termed focal adhesions and hemidesmosomes. In the activated, or extended conformation, they are more frequently localized at focal adhesions than in other regions of the plasma membrane (Askari *et al.* 2010), and the most common integrin found in focal adhesions of vertebrates is αVβ3 (Karp 2015). Focal adhesions are areas in the plasma membrane that contain large clusters of integrins that are connected intracellularly to actin filaments in the cytoskeleton. Because of the connection to both the extracellular matrix and the cytoskeleton, focal adhesions are dynamic structures that can assemble and disassemble depending on whether the cell is adherent, in motion, or entering mitosis (Karp 2015). Another region of integrin clusters is at the basal surface of polarized epithelial cells in structures termed hemidesmosomes, which link the cells to the basal lamina. These are similar to focal adhesions in that they are regions where integrins are attached to the cytoskeleton, but interactions with the cytoskeleton are through keratin filaments, or intermediate filaments, rather than actin filaments (Karp 2015). Integrins located in hemidesmosomes form the tightest attachment points between cells and their extracellular matrix (van der Flier & Sonnenberg 2001).

Integrins are found in a wide range of both simple and complex metazoans, but no homologues have been found in prokaryotes, plants, or fungi (Whittaker & Hynes 2002). In mammals, 24 integrins have been identified that bind a large number of ligands, including soluble and surface-bound proteins (Arnaout *et al.* 2005). Integrins are involved in modulating many of the cell's metabolic and signal transduction mechanisms (Ingber 2003). There are many

different integrins within and among organisms, each one with a specific distribution within the body. Most integrins are present on many different cell types, and conversely, many cells possess a variety of different integrins. Integrin diversity is further increased by alternative splicing, post-translational modification, and interaction with other intracellular and cell surface molecules (Plow *et al.* 2000). Because of the high diversity of integrin expression and distribution on cells, it is possible that integrins play a key role in determining cell phenotypes. Cell types typically possessing integrins include muscle, fibroblast, neuronal, epithelial and endothelial cells, as well as many cells in the embryo (Neff *et al.* 1982; Chen *et al.* 1985; Bozyczko & Horwitz 1986).

Integrins function as mechanochemical sensors and transducers and are the major receptor for cell adhesion to the extracellular matrix, but they can also mediate cell-cell interactions (Arnaout *et al.* 2005). They also provide a connection to the intracellular environment to perform a variety of signalling functions. These processes are critical in growth, development, and homeostasis (Arnaout *et al.* 2005). They are involved in modulating many of the cells' metabolic and signal transduction mechanisms (Ingber 2003) and aid in cell adhesion, migration, proliferation, growth, differentiation, immune signalling, and survival (Reviewed in: Karp 2015; Kinashi 2012; Chao & Kunz 2009; Evans *et al.* 2009; Carlos & Harlan 1994). Because cells may express many different integrins, they are capable of binding many ligands, although most integrins have unique functions (Karp 2015). Integrins recognize a large number of ligands, and because different cells express a variety of different integrins on the cell surface, many extracellular ligands are capable of binding to integrin-expressing cells. The main ligands of many integrins are proteins of the extracellular matrix such as fibronectin, various collagens, von Willebrand factor, vitronectin, and laminin (Karp 2015). Many of the extracellular proteins

that bind to integrins possess an arginine-glycine-aspartic acid (RGD) motif (Karp 2015), although not all integrin-binding ligands possess this motif.

Although ligand-receptor interactions play key roles in many of the body's normal functioning, including cell migration, development, and tissue maintenance (Chao & Kunz 2009; Caswell & Norman 2008), sometimes pathogens such as viruses take advantage of these transmembrane receptors as a means of attachment and subsequent entry into host cells, or to move between cells (Mercer *et al.* 2010). For example, *Yersinia enterolitica* has been shown to utilize β1 integrin receptors to adhere to and gain entry into epithelial cells (Hudson *et al.* 2005). Additionally, WNV binds to $\alpha_V\beta_3$ integrin on African green monkey kidney cells, and in particular, the β3 subunit played a necessary role in WNV binding and penetration into cells, as demonstrated through receptor competition assays using functional blocking antibodies against αV and β3 integrin subunits (Chu & Ng 2004). Interestingly, when other flaviviruses, specifically Japanese Encephalitis virus (JEV) and Dengue virus (DV), were tested against αVβ3 integrin using the same receptor competition assay, inhibition of JEV entry into Vero cells was similar to WNV but DV entry was only partially blocked, indicating that αVβ3 integrin is specific in mediating entry of WNV and JEV (Chu & Ng 2004). Additionally, soluble αVβ3 integrin blocked WNV entry into Vero cells and recombinant αVβ3 integrin expressed on a number of vertebrate cell types increased susceptibility of these cells to WNV infection (Chu & Ng 2004). The specific interaction between WNV and αVβ3 integrin was also shown to activate the outside-in integrin-associated signal transduction pathway necessary for viral entry into host cells (Chu & Ng 2004). These experiments further confirmed the specificity of αVβ3 integrin in mediating WNV entry into Vero cells. Because integrins are found in all metazoans (invertebrates to vertebrates), and because they are utilized as receptors for certain pathogens, it

is worthwhile exploring the possible role of an αVβ3 integrin homologue in WNV infection in the primary host organisms, birds.

The specific objectives of my research were to:

1) Compare partial gene sequences of αVβ3 integrin in several different bird species and determine their level of homology, as similar molecules may play similar roles in the WNV infection process among different bird species.

2) Examine expression levels of WNV in different bird species and tissues to help provide insight into the levels of virus within key tissues in different bird host species.

3) Examine expression levels of αVβ3 integrin in heart, liver, kidney, and brain tissues in crows and sparrows. Differences in expression levels among key tissues may be related to differential infection rates and/or host mortality due to WNV infection.

4) Determine the relationship between the expression levels of αVβ3 integrin in WNV-infected and non-infected birds in order to determine if there is a correlation in the abundance of these cell surface molecules and the infection status of a bird.

2.2 Methods

2.2.1 αVβ3 Integrin Sequence Homology among Different Bird Species

Birds, kindly provided by Dr. Terry Galloway, were obtained from the Manitoba Wildlife Rehabilitation Organization, Glenlea, Manitoba, and were held under scientific permits issued by

the Canadian Wildlife Service (CWS99-M023; 13-MB-SC001). All birds were casualties of various accidents or infections and had died or were euthanized at the rehabilitation hospital. Dissections of heart, liver, kidney, and brain tissues were performed on 30 American crows (*Corvus brachyrhynchos*) and 23 house sparrows (*Passer domesticus*). By creating multiple sequence alignments using known gene sequences of *Homo sapiens*, *Gallus gallus*, *Meleagris gallopavo*, and *Taeniopygia guttata,* gene-specific degenerate primers were designed to target the internal regions of the β3 integrin gene as well as a ribosomal protein gene RPL27 to use as reference for quantitative reverse-transcriptase PCR (qRT-PCR) (Figures 2.2 and 2.3; Table 2.1). A BLAST search revealed that the primers designed to amplify the RPL27 and β3 integrin genes do not cross hybridize to other previously-identified genes within the family.

a)

```
G. gallus          GAAGACTACCCTGTGGACATCTACTACCTGATGGACCTGTCCAACTCCATGAAGGACGAT 1739
M. gallopavo       GAAGACTATCCCGTGGACATCTACTACCTAATGGACCTGTCCAACTCCATGAAGGATGAT 1739
T. guttata         GAAGATTACCCTGTGGACATCTACTACCTCATGGACCTGTCTAACTCAATGAAGGATGAT 1739
                   ***** ** ** ********************** *********** ***** ******** ***

G. gallus          CTGAAGAACATCCAGAACCTGGGTACCAAGCTGGCCAGTGAGATGCGCAAGCTCACCAGC 1799
M. gallopavo       CTGAGGAACATCCAGAACCTGGGTACCAAGCTGGCCAGCGAGATGCGCAAGCTCACCAGC 1799
T. guttata         CTGAGGAACATCCAGAACCTGGGCACAAAACTGGCCAGTGAGATGCGTAAGCTCACTAGC 1799
                   **** ****************** ** ** ******** ******** ******** ***

G. gallus          AACCTTCGCATCGGCTTTGGGGCCTTTGTGGACAAGCCCATTTCCCCTTACATGTACATC 1859
M. gallopavo       AACCTTCGCATCGGCTTTGGGGCCTTTGTGGACAAGCCCATTTCCCCCTACATGTACATC 1859
T. guttata         AACCTACGCATCGGCTTCGGGGCCTTTGTGGACAAACCCATTTCCCCATACATGTACATA 1859
                   ***** *********** ********************** *********** ***********

G. gallus          TCTCCTCCAGAAGCCATCAAGAACCCTTGCTATGAGATTGGGGAAAAGTGCTTGCCCATG 1919
M. gallopavo       TCTCCTCCAGAAGCCATCAGGAACCCTTGTTATGAGATCGGGGAAAAGTGCTTGCCCATG 1919
T. guttata         TCTCCTCCAGAAGCCATCAAGAACCCTTGCTATGAGATTGGGGAAACCTGCCTGCCCATG 1919
                   ******************* ********* ******** *******  *** ********

G. gallus          TTTGGATACAAACATGT 1936
M. gallopavo       TTTGGATACAAACATGT 1936
T. guttata         TTTGGGTACAAACATGT 1936
                   ***** ***********
```

b)

```
G. gallus     GAAGGTGGTGCTGGTGCTCGCCGGCCGCTACTCGGGGCGTAAGGCTGTCATCGTGAAGAA 109
M. gallopavo AATCTTGATGCTGGTG---GCTGGCCGCTACTCGGGGCGTAAGGCTGTCATCGTGAAGAA 80
T. guttata    GAAGGTGGTGCTGGTGCTGGCCGGCCGCTACTCCGGGCGCAAGGCCGTCATCGTGAAGAA 112
H. sapiens    GAAGGTGGTGCTTGTCCTGGCTGGACGCTACTCCGGACGCAAAGCTGTCATCGTGAAGAA 108
               *   ** **** **    ** ** ******** ** ** ** ** **************

G. gallus     CATCGACGATGGCACGTCTGATCGGCCCTACAGCCACGCCTTGGTGGCAGGCATCGACCG 169
M. gallopavo CATCGACGATGGCACATCCGACCGGCCCTACAGCCACGCCTTGGTGGCAGGCATCGACCG 140
T. guttata    CATCGACGATGGCACCTCGGACCGGCCGTACAGCCATGCCTTGGTGGCTGGCATCGACCG 172
H. sapiens    CATTGATGATGGCACCTCAGATCGCCCCTACAGCCATGCTCTGGTGGCTGGAATTGACCG 168
              *** ** ******** ** ** ** ** ******** **  ******* ** ** *****

G. gallus     CTACCCACGGAAGGTTACAGCAGCAATGGGCAAGAAGAAGATAGCGAAGAGGTCTAAGAT 229
M. gallopavo CTACCCGCGGAAGGTGACAGCGGCGATGGGCAAGAAGAAGATAGCAAAGAGGTCTAAGAT 200
T. guttata    CTACCCGCGTAAGGTGACTGCTGCCATGGGCAAGGAAAAGATCGCTAAAAGGTCCAAGAT 232
H. sapiens    CTACCCCCGCAAAGTGACAGCTGCCATGGGCAAGAAGAAGATCGCCAAGAGATCAAAGAT 228
              ****** ** ** ** ** ** ** ********* * ***** ** ** ** ** *****

G. gallus     CAAGTCTTTTGTGAAAGTTTACAACTACAACCACCTGATGCCCACCCGGTATTCTGTTGA 289
M. gallopavo CAAATCTTTTGTGAAAGTTTACAACTACAATCACCTGATGCCCACCCGGTATTCTGTTGA 260
T. guttata    CAAGTCCTTCGTGAAGGTTTACAACTACAACCACCTCATGCCCACTCGGTATTCTGTGGA 292
H. sapiens    AAAATCTTTTGTGAAAGTTTATAACTACAATCACCTAATGCCCACAAGGTACTCTGTGGA 288
               ** ** ** ***** ***** ******** ***** ********  **** ***** **

G. gallus     CATTCCCCTGGACAAAACTGTTGTCAATAAGGATGTGTTCAGGGACCCTGCTCTGAAACG 349
M. gallopavo TATTCCCCTGGACAAAACTGTTGTCAATAAGGATGTGTTCAGGGACCCTGCTCTGAAACG 320
T. guttata    TATTCCCCTGGACAAAACAGTGGTCAATAAGGACGTGTTCAGGGATCCTGCTCTGAAACG 352
H. sapiens    TATCCCCTTGGACAAAACTGTCGTCAATAAGGATGTCTTCAGAGATCCTGCTCTTAAACG 348
               ** *** ********** ** ********** ** ***** ** ******** *****

G. gallus     CAAAGCAAGACGTGAAGCTAAGGTGAAGTTTGAGGAGAGATACAAGACTGGCAAGAATAA 409
M. gallopavo CAAAGCAAGACGTGAAGCTAAGGTGAAGTTTGAGGAGAGATACAAGACTGGCAAGAATAA 380
T. guttata    CAAAGCAAGACGTGAAGCCAAGGTGAAATTTGAGGAGAGGTACAAAACTGGCAAGAATAA 412
H. sapiens    CAAGGCCCGACGGGAGGCCAAGGTCAAGTTTGAAGAGAGATACAAGACAGGCAAGAACAA 408
              *** **  **** ** ** ***** ** ***** ***** ***** ** ******** **

G. gallus     GTGGTTCTTCCAGAAGCTGCGATTCTAAATTTGAAATAGGACTGTTTCAATAAATGTTTA 469
M. gallopavo GTGGTTCTTCCAGAAGCTGCGATTCTAAATTTGAAATAGGACTGTTTCAATAAATGTTTA 440
T. guttata    GTGGTTCTTCCAGAAGCTGCGATTCTAAGGGTGTAACGAGGTTGCATCAATAAATGTTTA 472
H. sapiens    GTGGTTCTTCCAGAAACTGCGGTTTTAGAT--------GCTTTGTTT-------TGATCA 453
              *************** ***** ** **              **  *       ** * *
```

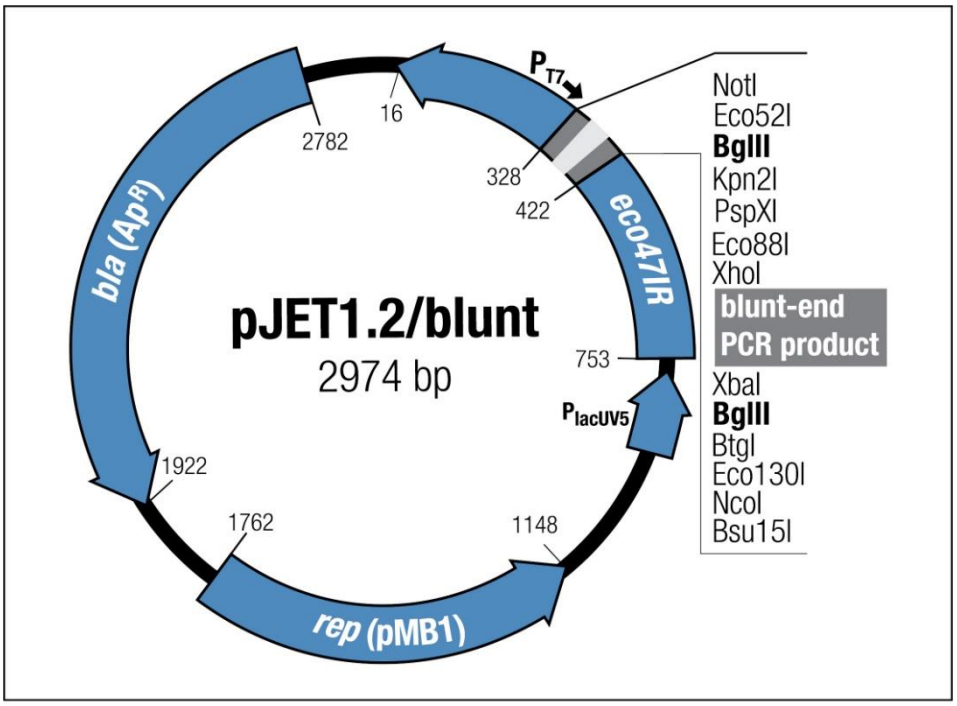
Figure 2.2. Multiple sequence alignment used to design degenerate primers for a) β3 integrin and b) RPL27 in birds. The highlighted sequences represent the regions of PCR primers.

Table 2.1. Degenerate primers used to amplify gene sequences in bird tissues.

Gene amplified	Primer sequence	Level of degeneracy	Size of fragment (nt)
RPL27	TCATCGTGAAGAACATYGAY	4	325
	TCTGGAAGAACCACTTRTTC	2	
Beta 3 integrin	YCCYGTGGACATCTACTACYT	8	243
	TTGTAGVCCAAACATGGG	3	

TRIzol (Life Technologies) RNA extractions were performed according to manufacturer's instructions on each of the bird tissues. Dissected tissues were placed in 1.5 ml microfuge tubes and crushed using a plastic pestle in 750 µl of Trizol. Extracted RNA was eluted in 50 µl RNase-free water and concentrations were determined using a Nanovue spectrophotometer (GE Healthcare). RNA was then treated with DNase I (Thermo Scientific) for use as a template for cDNA synthesis. cDNA was synthesized from 1 µg of the DNase-treated RNA samples using the qScript SuperMix cDNA synthesis kit (Quanta BioSciences). Samples were incubated at 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes, then cooled to 4°C for subsequent use in PCR reactions.

Degenerate primers for the β3 integrin and RPL27 genes from the two bird species were used in a standard 25 µl polymerase chain reaction (PCR) reaction (Econotaq, Lucigen) that was programmed as follows: 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 50.5°C for 30 seconds, 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. The PCR reactions were resolved on a 1.5% agarose gel supplemented with ethidium bromide and visualized using a bio imaging system (Syngene). Amplicons were excised from the gel and purified with Thermo Scientific's GeneJet Gel Extraction Kit according to manufacturer's instructions. Gel-purified samples were ligated into the pJET1.2 cloning vector (Thermo Scientific^{TM}, Figure 2.3) and the ligated plasmids were transformed into Subcloning Efficiency^{TM} DH5α Chemically Competent *Escherichia coli* cells (Invitrogen) using a heat-shock method as described by the manufacturer. The cells were plated onto LB agar plates (1% bacto-tryptone 0.5% bacto-yeast extract, 1% NaCl, 1.5% bacto-agar) substituted with 50 mg/ml ampicillin and incubated at 37°C overnight.



pJET1.2/blunt vector map.

Figure 2.3. Vector map for pJET1.2 blunt-end cloning vector (reproduced with permission from Thermo Fisher Scientific™).

Transformed bacterial colonies were screened for PCR amplicon inserts using a PCR screening method. Bacterial colonies were streaked onto a master plate and subsequently dipped into PCR tubes containing a standard PCR reaction mixture and plasmid-specific primers (5'-CGACTCACTATAGGGAGAGCGGC and 5'- AAGAACATCGATTTTCCATGGCAG). The cells were lysed by incubating the PCR tubes at 95°C for 10 minutes prior to PCR amplification to release the plasmid DNA templates. An annealing temperature of 63.7°C was used during the PCR cycles. Samples were resolved on a 1.5% agarose gel substituted with ethidium bromide to identify colonies containing the appropriately-sized insert fragments. Bacterial colonies were grown in a shaking incubator (225 rpm, 37°C overnight) in 3 ml of LB broth substituted with 50

mg/ml ampicillin. The plasmid DNA was isolated from the bacterial cells and purified using an E.Z.N.A® Plasmid Mini Kit 1 (Omega Biotek).

The identities of the sequences were analyzed by sequencing the amplicons (TCAG Sequencing Facility) and by comparing them to the genome databases available at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov) (Table 2.2). A Basic Local Alignment Search Tool (BLAST) was used to compare sequenced fragments to known gene sequences, and gene alignments were performed using Geneious software to confirm the identities of both the β3 integrin and RPL27 genes in both sparrows and crows.

Table 2.2: Gene sequence fragment lengths and percent identities of bird genes.

Gene	**Sequence Length (nt)**	**Percent pairwise identity to known sequences**	**Highest identity using a BLAST search (protein level)**
Crow-RPL27	381	78.0% to *Homo sapiens,* 82.4% to *Gallus gallus,* 89.1% to *Taeniopygia guttata,* 83.4% to *Meleagris gallopavo*	97% identity to *Taeniopygia guttata* ribosomal protein L27
Crow-β3 integrin	252	81.2% to *Homo sapiens* 89.1% to *Gallus gallus* 92.0% to *Taeniopygia guttata* 88.3% to *Meleagris gallopavo*	98% identity to *Taeniopygia guttata* integrin beta-3-like
Sparrow-RPL27	326	83.1% to *Homo sapiens,* 90.2% to *Gallus gallus,* 97.5% to *Taeniopygia guttata,* 90.8% to *Meleagris gallopavo*	98% identity to *Taeniopygia guttata* ribosomal protein L27
Sparrow-β3 integrin	293	81.3% to *Homo sapiens* 90.4% to *Gallus gallus* 90.8% to *Meleagris gallopavo* 96.4% to *Taeniopygia guttata*	98% identity to *Taeniopygia guttata* integrin beta-3-like

Maximum likelihood phylogenetic trees, using mosquito, other invertebrates, and vertebrate β3 integrin gene sequences were constructed using MEGA (6.06) to verify the identities of the gene targets (Appendix, supplemental Figure S2). The trees were tested using a bootstrap value of 1000 and the evolutionary distance of the phylogenetic tree was computed using a Poisson correction method in addition to eliminating gaps (Felsenstein, 1985).

2.2.2 Expression Levels of WNV in key bird host tissues

Gene-specific primers were designed for qRT-PCR gene expression analyses (Table 2.3) using the Beacon Designer™ program (Premier Biosoft). Primers were designed to produce amplicons fewer than 150 bp and have annealing temperatures within 1°C of each other. The ribosomal protein RPL27 gene was used as an internal reference gene for comparing quantities of WNV NS2A among the different bird tissues in both bird species.

Table 2.3. Gene-specific primers designed to target the NS2A region of West Nile virus.

Gene amplified	Primer sequence	Size of fragment (nt)
Crow RPL27	GTTTACAACTACAACCACCTG	87
	CCTGAACACATCCTTATTGAC	
Sparrow RPL27	GGGAATATCCACAGAATACC	78
	AGGTCCAAGATCAAGTCC	
WNV NS2A	GCCATAACATTCACAACGACATC	112
	CGACCATCAACAGCAGTATCC	

Using gene-specific primers to the non-structural protein WNV NS2a (Table 2.3) with RPL27 as the standard, quantitative reverse-transcriptase PCR (qRT-PCR) was used to determine whether WNV was expressed in any of the bird tissue samples. For each cDNA sample, qRT-PCR was performed in duplicate using a CFX Connect™ Real Time PCR Detection System. 96-well plates were set up with 15 µl reactions containing ~10 ng of cDNA, 7.5 µl of SsoFast™ EvaGreen® Supermix (BioRad), 1 µl each of 10 µM forward and reverse primers, and molecular grade water, using the following program: 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds and 59.4°C for 10 seconds, followed by a melt curve analysis to confirm that only a single PCR product was amplified.

The relative amount of WNV transcripts in the bird samples was determined using the $2^{-\Delta CT}$ method (Livak & Schmittgen 2001) where transcript levels are normalized to the internal standard (RPL27) using the following equation:

Fold change in WNV NS2A expression = $2^{-\Delta CT}$, where $\Delta CT = (CT_{WNV}-CT_{RPL27})$

Statistical significance of the data was assessed by performing unpaired t tests with a Welch correction.

2.2.3 Expression Levels of β3 Integrin in Heart, Liver, Kidney, and Brain Tissues among Different Bird Species in WNV-Infected and Uninfected Birds

Using β3 integrin gene-specific qRT-PCR primers (Table 2.3) designed by the Beacon Designer™ program (Premier Biosoft) and RPL27 primers to amplify the internal reference gene, qRT-PCR was performed on all bird tissues in both infected and uninfected sparrows and crows for gene expression analysis. Cycling conditions were performed as described in section 2.2.2 using a CFX Connect™ Real Time PCR Detection System with annealing temperatures of 52.4°C and 54°C in sparrows and crows respectively. The relative amount of β3 integrin transcripts in the bird samples was determined using the $2^{-\Delta CT}$ method and transcript levels were normalized to the internal standard RPL27.

2.3 Results

2.3.1 WNV expression in key bird host tissues

qRT-PCR was used to determine the presence of WNV in each of the heart, kidney, brain, and liver tissues of American crows and house sparrows. Ct values for RPL27 were within the conventional acceptable range (between 23 and 35) and were similar in both crows and sparrows using the same amount of RNA from similar tissues, thus the ribosomal protein gene was an effective reference for qRT-PCR. It was determined that 97% (29) of the 30 American crows collected were infected in at least one of the four tissues, whereas 91% (20) of the 23 house sparrows were infected. Interestingly, infected individuals did not necessarily show

detectable levels of WNV in all four tissues, and in some cases, only one or two tissues showed evidence of WNV infection (Figure 2.4, Table 2.4). A tissue-specific analysis of WNV gene expression revealed that 87% (26/30) of heart tissues were infected in crows and 73% (17/23) were infected in sparrows. In the kidneys, 81% (24/30) and 60% (14/23) of tissues were infected in crows and sparrows respectively. In the brain, 81% (24/30) and 39% (9/23) of tissues were infected in crows and sparrows respectively, and in the liver, 59% (18/30) of crows were infected and 39% (9/23) of sparrows were infected (Figure 2.4). Overall, a higher percentage of all four tissues were infected in crows relative to sparrows.

Table 2.4. Number of infected tissues in 30 American crows and 23 house sparrows.

	Crow	**Sparrow**
Heart, kidney, brain, liver	17	6
Heart, kidney, brain	7	4
Heart, kidney, liver	2	1
Heart, brain liver	0	2
Heart, kidney	0	3
Heart, brain	1	1
Heart, liver	0	3
Kidney, brain	0	0
Kidney, liver	0	1
Brain, liver	0	0
Heart	1	1
Kidney	0	0
Brain	1	0
Liver	0	0
None	1	2
Total birds:	30	23

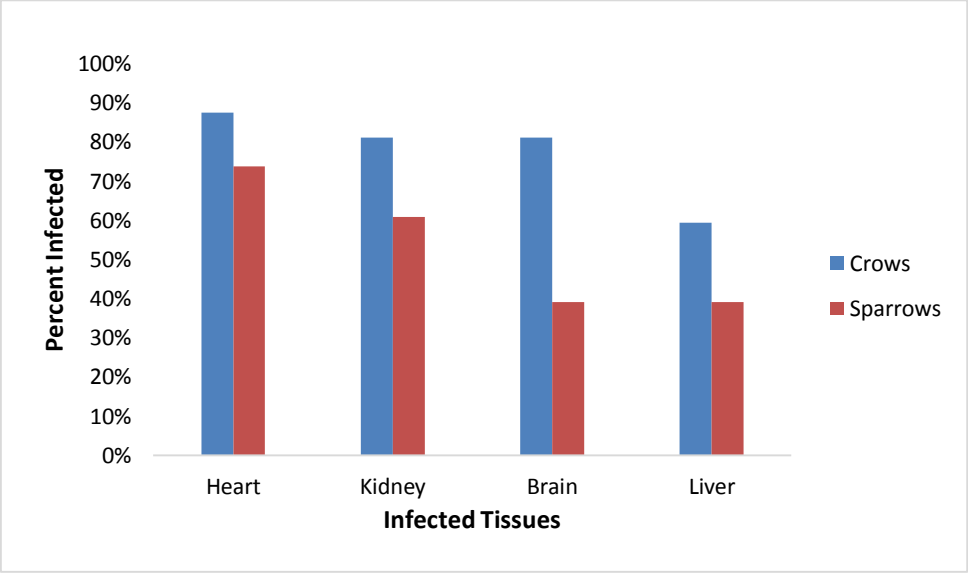


Figure 2.4. Percent of crows and sparrows infected with WNV from a total of 32 American crows and 23 house sparrows.

Using qRT-PCR to evaluate WNV gene expression relative to the ribosomal protein L27 reference gene in all four tissues, no statistical difference in infection rates could be found among the four tissues in the house sparrows ($P>0.05$). In the American crows, however, WNV expression was significantly higher in the heart than in the brain ($P<0.05$) (Figure 2.5). The large error bar in crow liver tissue indicates high variability in WNV transcript levels in that tissue within these samples. Interestingly, crows also had 400 to 6000 times more WNV transcripts in all four tissues relative to sparrows.

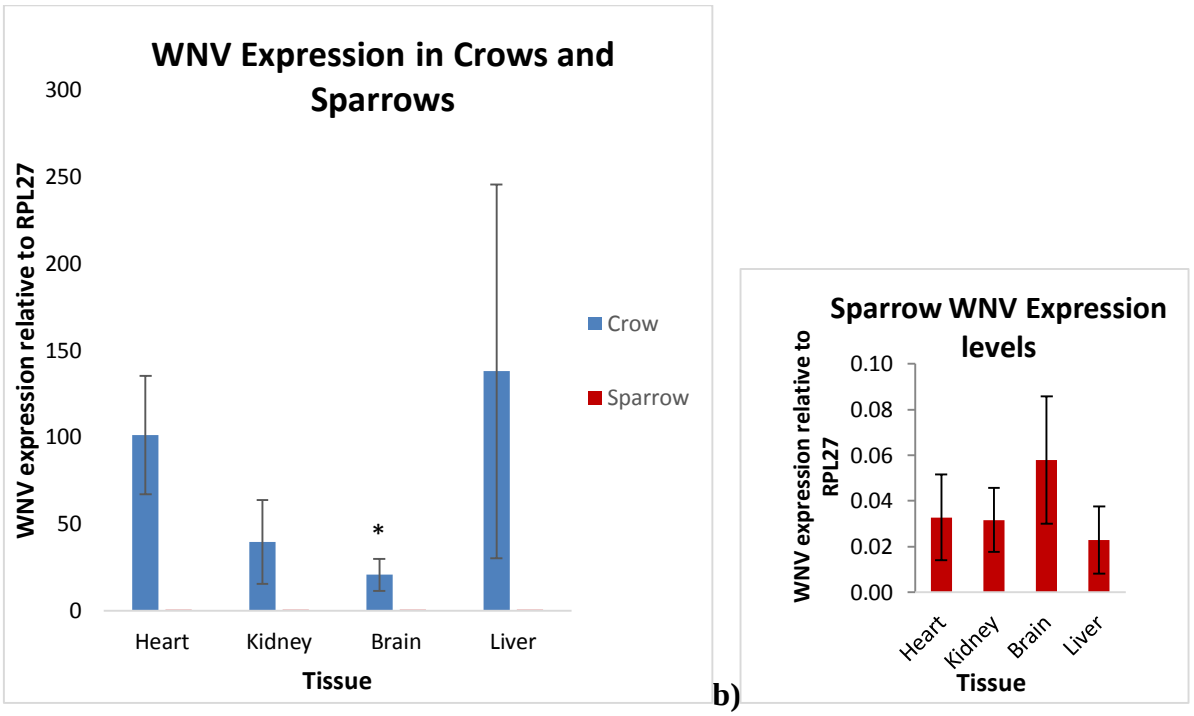
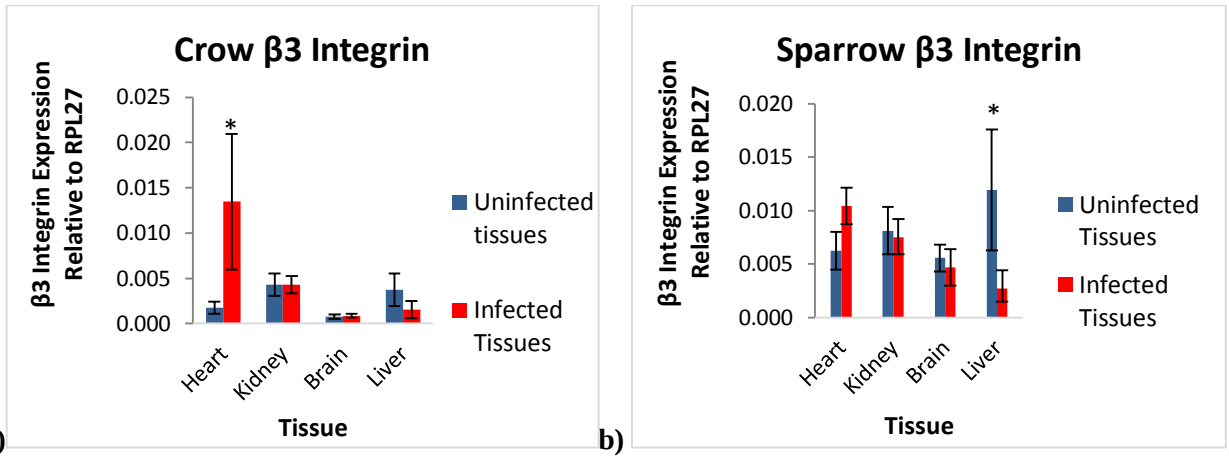


Figure 2.5. a) WNV expression in American crows relative to house sparrow heart, kidney, brain, and liver tissue. b) WNV expression house sparrow heart, kidney, brain, and liver tissue. Note the difference in the y-axes scales on the two graphs, as crows have much higher levels of WNV transcripts than sparrows.

Although the collection date was recorded for each bird specimen sampled, no correlation could be found between the infection level and date of collection (appendix, supplementary Figure S1). Additionally, the average levels of infection were not significantly different between the two years of sampling, therefore no year-to-year variation was observed. There was, however, a gap in sampling over the winter months, so the level of infection could not be determined over the course of the entire year in either species. Because of the lack of data over the winter months and the lack of variation within the summer months, it is not possible to determine which tissue is the typical first target for WNV during the course of infection.

2.3.2 β3 integrin expression in key bird host tissues

qRT-PCR was used to determine the transcript levels of β3 integrin in the heart, kidney, brain, and liver tissues in both American crows and house sparrows. Although crows displayed significantly higher WNV infection in all four tissues relative to sparrows, the range of integrin gene expression among all four tissues was relatively similar in both species (Figure 2.6). However, key differences in β3 integrin expression between certain infected and uninfected tissues were observed in both bird species. For instance, although no significant difference in β3 integrin expression was observed between uninfected and infected kidney, brain, and liver tissues in crows, integrin expression was significantly higher in the hearts of infected crows relative to uninfected crows ($P<0.05$; Figure 2.6 a). Additionally, integrin expression was significantly lower in both infected and uninfected brain tissue relative to heart and kidney tissues in the crows. In sparrows, β3 integrin expression was significantly higher in uninfected liver tissue relative to infected tissue (Figure 2.6 b). No other significant differences in gene expression were observed between infected and uninfected heart, kidney, or brain tissue in the sparrows. The large error bars in infected crow heart tissue and uninfected sparrow liver tissue indicates a high level of variation in β3 integrin transcript levels in these samples. When comparing the uninfected tissues between the two bird species, β3 integrin expression was significantly lower in crow heart and brain tissue than sparrows. No significant differences were found among infected tissues between the crows and sparrows.



a) **b)**

Figure 2.6. β3 integrin expression in infected and uninfected a) American crows and b) house sparrow heart, kidney, brain, and liver tissue.

2.4 Discussion

Birds are the primary hosts of WNV because viral titers in the blood are sufficient to infect mosquitoes when a blood meal is taken. Although WNV has been detected in over 300 different bird species in North America alone (CDC 2014; CCWHC 2014), members of the *Corvidae* family (such as American crows, blue jays, and magpies) and the *Passeridae* family (such as house sparrows) are considered to be the main reservoir hosts in North America and Europe (Kilpatrick *et al.* 2006; Langevin *et al.* 2005). Some species carry the virus but remain asymptomatic whereas other species are particularly vulnerable to infection and develop clinical signs, and it is currently unknown why this variability in pathology among different bird species exists.

WNV infects all major organ systems and most cell types, and although the viral replication and infection pathway is known in mammals, the mechanism and primary sites of infection are unknown in bird hosts. Furthermore, the mechanism by which WNV crosses the blood-brain barrier and enters the CNS to cause encephalitis remains elusive. It has been suggested that changes in endothelial cell permeability or transport of the virus in infected

immune cells could facilitate entry into the brain and CNS (Wang *et al.* 2004; Garcia-Tapia *et al.* 2006). WNV can infect and replicate within mononuclear phagocytic (MP) cells, and therefore, these cells have been implicated in aiding the dissemination of the virus to various tissues (Garcia-Tapia *et al.* 2006; Weingartl *et al.* 2004). Furthermore, HIV-1-infected MP cells have been shown to weaken tight junctions in brain microvascular endothelial cells and impair blood-brain barrier function in murine models, leading to encephalitis (Persidsky *et al.* 2000). Although this method has not yet been explored for WNV in avian systems, it is possible that a similar route of infection and mechanism of virally-induced pathology exists in birds. Additionally, integrins play a role in regulating immune responses by aiding the migration of leukocytes to lymph nodes and sites of injury, and by facilitating the adherence of leukocytes to endothelial cells (Reviewed in: Kinashi 2012; Evans *et al.* 2009; Carlos & Harlan 1994). Changes in integrin levels may reflect immunity-based host cell responses to WNV infection in order to initiate host cell protection mechanisms, for instance, an increase in integrin expression may enhance cell-cell adhesion, thus reducing the permeability of tissues to WNV infection.

Rapid dissemination of the virus throughout the body and the penetration of the blood-brain barrier could occur more easily in some bird species than others, and could explain why certain species, such as crows, have higher mortality rates than others. The birds collected for my experiments were obtained from wildlife conservation and rehabilitation centres and were already deceased upon receipt; the cause of death for each of the birds was unknown. Although no sampling of dead birds occurred over the winter months, WNV infection of wild birds typically begins in the spring to early summer and the highest mortality is seen during mid-summer to early fall (Phalen & Dahlhausen 2004). This could explain the high percentage of infected birds of both species observed in this relatively small sample size (Figure 2.4). It is

possible that many of the birds used in my study died from WNV infection, and hence were more readily collected by bird naturalists. The high proportion of infected individuals (97% and 91.3% in crows and sparrows respectively) supports this theory, although it does not explain the 400-6000 fold difference in WNV transcript levels between crows and sparrows (Figure 2.4; Figure 2.5). The high WNV transcript levels detected in crow tissues relative to sparrow tissues may explain the high mortality typically seen in crows (Langevin *et al.* 2005; Brault *et al.* 2004), but further studies will be required to explore the correlation between WNV titers and mortality rates in these birds. The low levels of WNV in sparrows relative to crows is inconsistent with other findings that demonstrate high levels of virus in experimentally-infected sparrows (Komar 2003), although Del Amo *et al.* (2014) found that viremia was significantly lower in sparrows that survived WNV infection. This could indicate an immunity to the virus or a heightened ability to clear the virus because those sparrows also tended to be asymptomatic. Langevin *et al.* (2005) also found that sparrows that survived WNV infection all developed neutralizing antibodies, supporting the theory that sparrows develop an immunity to the virus. Alternatively, infections by different strains of WNV produce differences in the amount of virus circulating in the blood, neuroinvasiveness, and mortality in sparrows (Langevin *et al.* 2005); however in North America, the NY99 strain is the primary form of WNV and differences in WNV strains would not account for differences in viremia in this population.

The timing of the initial exposure to the virus and the duration of viral infection in the birds in this study are, unfortunately, not known. If the birds were infected shortly before collection, it is possible the virus had not yet spread throughout the entire body, and neural tissues would not necessarily be infected. This could explain why only 14 of the 30 American crows (46.6%) and 6 of the 23 house sparrows (26.0%) had detectable levels of WNV in all four

key tissues (heart, brain, kidney, and liver) (Table 2.4). Interestingly, brain infections were more prevalent in the crows than in the sparrows in my study (Table 2.4; Figure 2.4), but this may not be the causative factor for death, as Steele et al. (2000) found that death by infection in the brain is less frequent in crows and magpies than other bird species. It is possible that the primary cause of death due to WNV is not related to neuroinvasiveness in birds and instead, mortality is due to other factors such as myocardial stress and heart failure (Swayne *et al.* 2001). WNV gene expression was significantly higher in crow heart tissue relative to brain tissue, which could support this theory (Figure 2.5). Alternately, it could indicate a high presence of WNV in the blood and circulatory system, causing systemic infection, which could explain why all four tissues were infected at a higher percentage of crows than sparrows. Because sparrows did not display any significant differences in WNV infection among all four tissues, this, along with the overall low levels of WNV the tissues, could explain why mortality due to WNV infection is reportedly low in this species relative to crows (Figure 2.5; Del Amo *et al.* 2014).

High WNV levels in crows relative to sparrows could be due to the amount or type of cell surface receptors found in key tissues, such as the heart or brain. Because integrin is a putative host cell receptor for WNV (Chu & Ng 2004), integrin gene expression levels were measured using qRT-PCR to determine whether or not there were differences between the two species. Interestingly, although crows displayed much higher levels of WNV, β3 integrin transcript levels were much less variable between crows and sparrows (Figure 2.6). This lack of correspondence between WNV titers and β3 integrin transcript levels could indicate that this particular integrin is not one of the key host cell receptors, or that gene expression of this putative receptor is not altered by WNV infection. Measurements of the protein levels on the cell surface (rather than intracellular transcript levels) within the different tissues could help address this question.

However, integrin levels could also change during the course of infection, but due to the lack of information regarding the timing of infection of the birds, an examination of the protein levels was not considered a worthwhile pursuit. One notable difference in β3 integrin expression was observed in the hearts of the crow: there was significantly higher expression in infected crow hearts relative to uninfected hearts (Figure 2.6). This could account for the high levels of WNV in the heart tissue if, in fact, the virus utilizes this integrin as a host cell receptor. If the virus bound to the integrin protein and perhaps interfered with its normal functions, the cell may respond to WNV binding by producing more β3 integrin to maintain the integrity of cell adhesion. If the junctions between cells became compromised due to a lack of integrin, tissue membranes could become more permeable to foreign substances and tissue damage could occur (Robinson *et al.* 2004). If this were to occur in the heart, an increased risk of death due to heart failure is possible. In contrast, integrin could be modulating the immune response by facilitating the adhesion of leukocytes to endothelial cells, and an increase in integrin could indicate the activation of a host defense mechanism. A low level of integrin gene expression, as seen in both the infected and uninfected brain tissues in crows, could be related to the low levels of virus found in the brain (Figure 2.6). If there are fewer integrin proteins expressed in cells in the brain, there would be fewer receptors for which the virus is able to bind, and therefore a lower infection level could be seen in the brain. Additionally, if fewer virus particles bound to the integrin proteins, there would be a diminished need to produce new proteins to replace those that were compromised, and therefore integrin gene expression would be low. This model contrasts the theory that high levels of integrin in brain tissue results in increased cell-cell adhesion, thus reducing permeability of the blood-brain barrier to infectious pathogens. Due to the lack of information regarding how WNV interacts with integrin in the brains of birds, more than one

interpretation of these data is possible.

Low integrin expression was found in infected liver tissues relative to uninfected liver tissues in sparrows, which could indicate an internal defence mechanism to prevent further infection of compromised tissue and increase survival (Figure 2.6). Additionally, integrin expression was significantly lower in uninfected crow heart and brain tissues relative to sparrows, but no significant differences could be seen in infected tissues. Given that WNV expression was significantly higher in all four tissues in crows relative to sparrows yet integrin expression was relatively uniform, the amount of integrin expression may not really play a prominent role in differential WNV infection rates between the two species. Additionally, the variability in integrin expression between infected and uninfected tissues in both species was extensive, which provides no support to the hypothesis that β3 integrin expression is a primary regulator of WNV infection in birds.

Unfortunately, the data do not provide any insights into the role of β3 integrin in WNV infection in birds. Without records on the time of death, no definitive conclusions concerning the seasonality of the viral infections can be determined. Also, the birds were of unknown ages and the juveniles were of unknown sex (as gonadal development is not evident in young birds), making it impossible to determine whether WNV infections were more prevalent in certain ages or sexes of the birds. To fully understand whether WNV infections are associated with integrin expression, it would be ideal to infect lab-bred birds and to monitor the course of infection and gene expression, although considerable time would be needed to acquire the permits and to quarantine the animals in a high security facility for such studies. Although there were limitations with the samples available, this experiment has provided some insight into the extent of infection in different bird species as well as the distribution of the virus within bird hosts.

Chapter 3: Examining the role of putative susceptibility and resistance factors for WNV infections in mosquitoes

3.1 Introduction

Mosquitoes are known as the most deadly animals on earth (Gates 2014), annually infecting approximately 700 million people worldwide with parasites and viruses that cause diseases such as malaria, dengue fever, Japanese encephalitis virus, and West Nile virus, which together, result in over 1 million fatalities each year (Carabello & King 2014). Due to their role as disease vectors, mosquitoes have been and continue to be the focus of many biological control programs that aim to reduce the numbers of disease-causing species in order to limit the spread of infections between human hosts (reviewed in: Whyard *et al.* 2015; Oxitec 2012; Alphey *et al.* 2009; Scholte *et al.* 2005). With the relatively recent introduction of WNV into the western hemisphere, increased efforts have been made to control the spread of the virus through surveillance programs of infected hosts, mosquito control, and vaccine development (NIH 2015; PHAC 2015; CDC 2009). Prevention of contracting the virus is a crucial step towards limiting the number of clinical cases, and because different species of mosquitoes have varying abilities to transmit the virus, identifying vector competence as well as factors contributing to their ability to transmit the virus are critical to developing suitable surveillance and control strategies. Currently, *Culex* spp. are the primary global vectors of WNV, with species such as *Cx. tarsalis* and those of the *Cx. pipiens* complex, including *Cx. quinquefaciatus*, serving as the dominant vectors in Canada and the US. Although mosquitoes of other genera including *Aedes*, *Anopheles*, *Coquillettida*, *Culiseta*, and *Ochlerotatus*, can become infected with WNV, their abilities to transmit the virus are considerably inferior (Cheng *et al.* 2010; CDC 2012; Turell *et al.* 2005; Sardelis *et al.* 2001; Turell *et al.* 2000). The reasons for these differences in susceptibility to

infection and capability to transmit the virus remain unclear; however differences in the mosquitoes' immune responses as well as differentially regulated genes and proteins in key tissues may play a role in enhancing or reducing vector competence.

In a previous study, Krishnan *et al.* (2008) conducted a human genome-wide screen that identified 305 human protein-encoding genes that affected WNV infection, and a follow-up study identified 215 homologues of those genes in *A. aegypti* (Cheng *et al.* 2010). Of those 215 genes, 32 were affected by WNV in *A. aegypti* by either increasing or decreasing in transcript levels. RNAi-mediated knockdown of the 32 genes was subsequently conducted and viral load was assessed after mosquitoes were inoculated with WNV. Of the 32 genes, 13 were shown to increase or decrease the viral load in *A. aegypti*. One of the 13 genes was identified as a C-type lectin, termed mosGCTL-1, and another was identified as a CD45 homologue, both of which were examined more thoroughly because WNV levels decreased the most when those genes were knocked down relative to all other genes in the study. Cheng *et al.* (2010) determined that these two proteins interacted directly with the E-glycoprotein of WNV, and therefore they believed that the two proteins were the most important factors in determining infections in the mosquitoes. Because of their conclusions, no such follow-up studies were performed on the other 11 genes; however because of the complexity of the mechanism by which WNV infects different hosts, it is probable that many factors play a role in WNV attachment and entry into host cells, replication, and release from cells. The 13 genes were subdivided into resistance factors, which are host cell proteins that reduce WNV infection, and susceptibility factors, which are host cell proteins that facilitate WNV infection. There is currently little understanding of how these proteins might mediate resistance or susceptibility, but what is currently known of their predicted cellular functions is indicated in Table 3.1. Two clathrin coat adaptor proteins were identified in

this study as influencing WNV infection in *A. aegypti* mosquitoes; however, interestingly, one was acting as a susceptibility factor (AP1) whereas the other was acting as a resistance factor (AP3). Further exploration of their precise roles in mediating these contrasting roles in WNV infections is warranted.

Table 3.1. a) Resistance factor genes and b) susceptibility factor genes and their predicted functions (derived from NCBI and VectorBase). Accession numbers for *A. aegypti* are listed as follows: VectorBase accession number of the gene, NCBI reference number of the gene, and NCBI protein sequence number.

a)

Accession number in *A. aegytpi*	**Accession number of *Cx. quinquefasciatus* orthologue**	**Percent Identity (protein)**	**Gene Name**	**Predicted Functions**
AAEL001405 XM_001659143.1 XP_001659193	XP_001866129.1	99%	Clathrin coat assembly protein AP3	Intracellular vesicle-mediated protein transport, link clathrin to receptors in coated vesicles, interact with cytoplasmic tails of membrane proteins leading to their selection and concentration, connect cargo proteins and lipids to clathrin at vesicle budding sites, protein trafficking to lysosomes and other related organelles.
AAEL013378 XM_001663509.1 XP_001663559.1	XP_001864921.1	94%	Hypothetical protein	Ion transport, cation-selective channels important for cellular calcium signalling and homeostasis
AAEL008358 XM_001653151.1 XP_001653201.1	XP_001850101	86%	AbLIM	Metal-binding, zinc ion binding, cytoskeleton organization, organ development and oncogenesis. protein: protein interactions
AAEL011844 XM_001661930.1 XP_001661980.1	XP_001866063	85%	5-hydroxytryptamine receptor 1 (5HT receptor, serotonin receptor)	Zinc ion binding, GPCR serotonin family, signal transducer activity, G-protein coupled receptor activity, ligand-gated ion channels in the central and peripheral nervous systems.
AAEL015099 XP_001647816.1 XM_001647766	XP_001850651	85%	Sumo ligase	Adds the SUMO tag (SUMO=small ubiquitin-like moiety), nucleic acid binding, zinc ion binding, protein stability, nuclear-cytosolic transport, transcriptional regulation.

AAEL000434 XM_001656414.1 XP_001656464.1	XP_001844399.1	78%	Lipid A export ATP-binding/permease protein msba	ATP-binding, ATP-ase activity, nucleoside triphosphatase activity, integral to membrane, coupled to transmembrane movement of substances.
AAEL014547 XM_001648919.1 XP_001648969.1	XP_001848278	68%	Hypothetical protein partial mRNA (*Aedes aegypti*) Sushi (*Culex quinquefasciatus*)	Protein binding, EGF-like domain, disulfide bond, complement control protein (CCP) modules, or short consensus repeats (SCR), blood group antigens.

b)

Accession number in *A. aegytpi*	**Accession number in *Cx. quinquefasciatus***	**Percent Identity (protein)**	**Gene Name**	**Predicted Function**
AAEL007124 XP_001652559.1. XM_001652509.1.	XP_001868211	99%	Clathrin coat assembly protein AP1	Intracellular protein transport, vesicle-mediated transport, protein binding, assembly of clathrin-coated vesicles originating from the trans-Golgi network.
AAEL012094 XP_001655862.1 XM_001655812.1.	XP_001861863	99%	Casein kinase ii, alpha chain (cmgc group iv)	Nucleotide-binding protein, serine/threonine kinase activity, ATP binding, protein phosphorylation, transfers phosphorous-containing groups, cell cycle control, DNA repair
AAEL004574 XP_001649382.1. XM_001649332.1.	XP_001845048.1	91%	SAGA-associated factor 11 homolog (SGF11)	Component of the transcription regulatory histone acetylation (HAT) complex SAGA, activates transcription by remodeling chromatin and mediating histone acetylation and deubiquitination.
AAEL007039 XP_001658085.1 XM_001658035.1.	XP_001867002.1	86%	PGRPS5b, Peptidoglycan recognition protein sc2	Peptidoglycan catabolic process, N-acetylmuramoyl-L-alanine amidase activity, zinc ion binding.
AAEL014037 XP_001657325.1. XM_001657275.1.	XP_001852246.1	78%	PAFacetylhydrolase 45 kDa subunit (*Aedes aegypti*) WD repeat protein 18 (*Culex quinquefasciatus*)	Protein binding, enzyme that catabolizes platelet-activating factor, functions as a component of the Five Friends of Methylated CHTOP (5FMC) complex
AAEL000563 XP_001647954.1. XM_001647904.1.	XP_001867205.1	48%	Galactose-specific C-type lectin (mosGCTL-1)	Carbohydrate binding, protein binding

Clathrin coat adaptor proteins are involved in the endocytic process within eukaryotic cells and because this is the mode of entry of WNV into cells, they could influence the extent of viral infection. Endocytosis is the process by which cells engulf molecules found at the cell surface. To do so, an invagination forms at the plasma membrane that pinches off from the plasma membrane through scission to form a vesicle. These vesicles are subsequently transported throughout the cell and fuse with the target membranes of early endosomes to become further processed into late endosomes and subsequent lysosomes for protein degradation, or recycling endosomes for transport of cargo proteins back to the cell surface (van Ijzendoorn 2006). This process is necessary for nutrient uptake, regulation of transmembrane receptors, and vesicle recycling. It is divided into three primary categories: phagocytosis, a process where solid particles become engulfed within the cell, pinocytosis, where extracellular fluid and solutes are engulfed, and receptor-mediated endocytosis, where particles bind specifically to receptors on the cell surface prior to being taken up by the cell (Popova *et al.* 2013; Flannagan *et al.* 2012; Nakatsu *et al.* 2003; McPherson *et al.* 2000). The latter of the three is the process by which WNV gains entry into susceptible cells. Receptor-mediated endocytosis can further be sub-divided into more specific categories that include clathrin-mediated internalization, caveolin-mediated internalization, and clathrin- caveolin-independent internalization (Schütze *et al.* 2008). The latter two depend upon lipid rafts which are cholesterol-rich portions of the plasma membrane that also contain glycosphingolipids and GPI-anchored membrane proteins (Pelkmans 2005). Clathrin-mediated endocytosis (CME) is found in all known eukaryotic cells and involves the binding of external ligands to transmembrane receptor proteins in the plasma membrane, followed by budding of the plasma membrane containing the bound ligand into clathrin-coated vesicles (McMahon & Boucrot 2011; McPherson *et al.* 2009; Popova *et al.* 2013). This multi-

step process involves more than fifty different proteins including adaptors that link transmembrane cargo proteins to the polymerized clathrin coat, scission factors that aid in actin polymerization, and proteins that facilitate un-coating of the vesicle (McPherson *et al.* 2009; Popova *et al.* 2013). There are three layers in a clathrin-coated vesicle: the outer layer of polymerized clathrin, an internal lipid layer that also contains bound proteins, and a layer of adaptor proteins that binds clathrin to the lipid-bound integral proteins of the forming vesicle (Popova *et al.* 2013).

Adaptor protein (AP) complexes are cytosolic, heterotetrameric structures that consist of two large chains of adaptins (β and γ or α depending on the type of protein) which form the core of the structure, along with two appendage domains that are made up of a medium (μ) and a small (δ) chain of adaptin (Figure 3.1; Heldwein *et al.* 2004; Lefkir *et al.* 2003; Pearse *et al.* 2000) The β and γ (or α) chains are responsible for binding to the cytoplasmic tails of cargo-binding proteins (i.e. receptors) in order to select, sort, and concentrate them into budding vesicles, and the μ and δ chains bind to clathrin and accessory proteins (Ohno *et al.* 2006; Lefkir *et al.* 2003). There are four main groups of adaptor proteins, each with varying functions and designated locations within the cell. AP1 is localized in the trans-Golgi network (TGN) and acts to transport lysosomal hydrolases and proteins from the TGN to endosomes, as well as aiding the transport of endosomes back to the TGN (Lefkir *et al.* 2003; Nakatsu & Ohno 2003). Because of its association with the TGN, it has also been implicated in aiding the process of exocytosis (Nakatsu & Ohno 2003). The AP2 adaptor complex binds to the plasma membrane and aids in the budding process of endocytosis by triggering the formation of clathrin lattices at sites where cargo proteins are found (Nakatsu & Ohno 2003; Popova *et al.* 2013). AP3 is associated with the TGN as well as with endosomes and is involved in the transport of membrane proteins to

lysosomes (Gupta *et al.* 2006; Ihrke *et al.* 2004; Nakatsu & Ohno 2003). Less is known about AP4, although it may also be involved in trafficking of proteins from the TGN to endosomes (Gupta *et al.* 2006). Because the AP1, AP3, and AP4 adaptor complexes are associated with the membranes of endosomes and the TGN, they also play a role in exocytosis by regulating protein traffic in the post-Golgi network (Jaiswal *et al.* 2009; Vassilieva & Nusrat 2008, Gupta *et al.* 2006, Nakatsu & Ohno 2003; McPherson *et al.* 2000). AP1 and AP4 in particular are involved in trafficking vesicles containing newly synthesized proteins from the TGN to the plasma membrane in addition to late endosomes and lysosomes (Nakatsu & Ohno 2003). Interactions of the AP proteins with the endomembrane system are complex and involve many different signalling molecules for their regulation. For this reason, mechanisms of how different external molecules become processed within the cell are still elusive, particularly during the infection process of viruses and other pathogens.

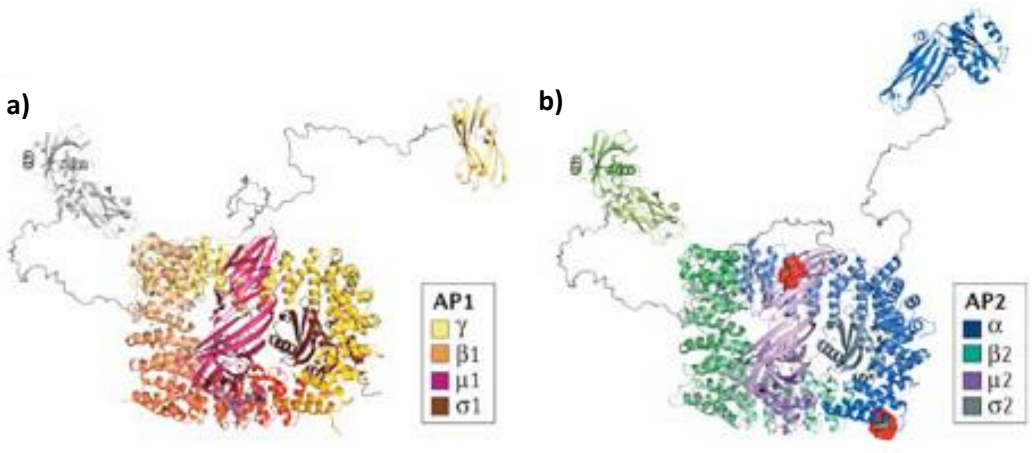


Figure 3.1. X-ray crystallographic images of the protein structure of clathrin coat adaptor proteins a) AP1 and b) AP2. (Protein Data Bank, accession files 1W63, 1GYU, 1GW5, 1B9K, and 1E42)

The natural roles of CME within cells include the uptake of nutrients and the turnover of plasma membrane proteins, but because of its ubiquity, pathogens, including viruses and bacterial toxins, are also able to utilize this pathway to gain entry into cells (Vassilieva & Nusrat 2008; Popova *et al.* 2013; McPherson *et al.* 2000). WNV, like all Flaviviruses, enter the cell through CME, presumably because the proposed receptors for the E glycoprotein ligand include molecules such as integrins and C-type lectins, both of which utilise the CME pathway to become internalized into the cell (Smit *et al.* 2011; Cambi *et al.* 2009). It is possible that differential regulation of the factors involved in CME influences viral uptake, transport, replication, and/or release from infected or susceptible cells. One mechanism of manipulation of cellular machinery includes RNA interference (RNAi). RNAi is a process that was first discovered by Andrew Fire and colleagues in 1998 in which mRNA transcripts are silenced by a sequence-specific double stranded RNA (dsRNA) molecule (Fire *et al.* 1998). When a long strand of dsRNA enters a cell, it binds to an RNAse II-like enzyme called Dicer which cuts the dsRNA molecule into small (21-23 nucleotide) fragments called small interfering RNAs (siRNA) (Campbell & Choy 2005). These siRNA molecules associate with an RNA-induced silencing complex (RISC), which together target and cleave complementary sequences on mRNA transcripts, ultimately reducing the expression of a particular gene (reviewed in Hannon 2002). By knocking down gene expression, presumably, protein expression will also be reduced, although depending on the method used to obtain gene knockdown, factors such as the duration of protein turnaround and half-life will also influence the effectiveness of RNAi. With the knockdown of AP complex genes, their roles in WNV infection of cells will be better elucidated, and by determining their effects on WNV infections in different mosquito species, we will gain a better understanding of the mechanisms of viral infection and vector competence.

The aim of this study was to analyze gene expression of two different resistance factor and susceptibility factor genes in different mosquito species and tissues, and determine if WNV is affected by those genes in different species in order to gain a better understanding of the differences in vector competence. Specifically, my objectives were to:

1) Examine expression levels of key genes previously identified as having played a role in WNV infection (Cheng *et al.* 2010) among different mosquito species, particularly, known vector species for WNV and species that do not typically act as vectors. This may provide insight into the relevance of these cell surface molecules in mosquito-vector competence.

2) To suppress the expression of certain key genes in mosquito cell cultures using RNAi and determine whether knockdown of the genes affects the virus's ability to bind to and infect the cells. This will help to identify the relevance of these genes in WNV infection.

3.2 Methods

3.2.1 Mosquito Rearing

Culex quinquefasciatus eggs were kindly provided by Mahmood Iranpour from the National Microbiology Laboratory and were hatched and reared in the lab of Dr. Steve Whyard, along with a colony of McAllen strain *Aedes aegypti*. Mosquitoes were reared at 28°C with 50% humidity, on a 16h light: 8h dark photoperiod. Eggs were hatched by placing them in small tubs of dechlorinated tap water (500 ml) with gerbil food pellets (1/tub). Ground, desiccated liver tablets were mixed with water (1 mg/50 ml) and incubated overnight at 37°C shaking at 250 rpm. The liver powder mixture was added to the tubs (~2 ml/L) to induce hatching. Larvae were

maintained in these tubs until pupation. Pupae were then collected and placed in individual vials to eclose. Adults were maintained on a 10% sucrose solution until the time of dissection.

To maintain the *A. aegypti* colony, pupae were placed in a large cage and maintained with a 10% sucrose solution. Females were blood fed once a week by creating a "blood bag" from stretched Nescofilm (Karlan Research Products) containing ~2 ml of 42°C blood obtained from rats maintained in the Animal Holding Facilities of the University of Manitoba, Fort Garry Campus. Dishes lined with dampened paper towels were placed in the cage where females were allowed to lay eggs. Eggs were stored in a humid 28°C incubator prior to hatching.

3.2.2 Expression Levels of Genes Affecting WNV in the Salivary Glands, Crop, Midgut, and Carcass of *Aedes aegypti* and *Culex quinquefasciatus*

Salivary gland, crop, midgut, and carcass tissues were dissected from 4-7 day-old adult *A. aegypti* and *Cx. quinquefasciatus* by teasing away tissues using fine forceps (Figure 1.1). Five biological replicates of 30-50 dissected tissues from each species were used for subsequent experimentation. RNA was extracted from the dissected tissues using a GeneJET RNA Purification Kit (Fermentas/Thermo Scientific) according to the manufacturer's instructions. First, mosquitoes were placed in 1.5 ml microfuge tubes and crushed using a plastic pestle in 300 µl of lysis buffer supplemented with 2% β-mercaptoethanol. The mixture was then placed in a QIAshredder (Qiagen) column to homogenize the tissues and the lysate was subsequently used with the RNA purification kit. Extracted RNA was eluted in 30 µl of RNase-free water twice, and concentrations were determined using a (Nanovue GE healthcare). RNA was then DNase-treated for cDNA synthesis as described in section 2.2.1.

Species- and gene-specific qRT-PCR primers for 6 resistance factor and 4 susceptibility factor genes identified by Cheng *et al.* 2010 were designed using NCBI's nucleotide database,

VectorBase (www.vectorbase.org), the Beacon Designer™ program (Premier Biosoft), and IDT's PrimerQuest® design tool (Table 3.2). These primers were used to perform qRT-PCR on the cDNA samples as described in section 2.2.2 using annealing temperatures 5°C lower than the primer with the lowest melting temperature. Data was analysed using the $2^{-\Delta CT}$ method as described in section 2.2.2, and transcript levels were normalized to the internal standard S7 ribosomal protein gene. Due to time constraints, only one each of the resistance factor and susceptibility factor genes were used in further experimentation: the clathrin coat adaptor proteins AP1 and AP3.

Table 3.2. qRT-PCR primers used to amplify genes in *Aedes aegypti* and *Culex quinquefasciatus*.

Gene	***Aedes aegypti***	**Amplicon size (nt)**	***Culex quinquefasciatus***	**Amplicon size (nt)**
ATP-binding cassette	CGATGGAGTAGCAGCAGTA	182	TGCTCCATCATCACCACCT	116
	GAAGAAAGCACCAAACAGC		AATCTTCCCCAAACCAAACG	
AbLIM	ACGACGCTATTCCGATTCC	147	CACGATGGGCAACACATA	109
	CCCATTCCCGATGACTTTAG		CTCGCACAGAACCTCCTT	
5HT receptor	ATCTTTGGATGGGTGGTTTG	142	ATTCGGAGAACACCCAGTA	154
	TGTTCTTCTTCGGTTTGAGG		AACAGCACCAGAAAGAACA	
Clathrin coat AP3	TGATGGGAGGTATGGTCCT	220	GATCGAAGAGCAAAACAAAC	130
	AAGTTCGTGTTTGGTCAGAA		GGGAAGATCAGGTAATTTGA	
Sumo Ligase	GCGGCTATCTACGACAATCT	176	AGCAGGAGGACTACTTTCC	137
	GACTTTTTGAACTGGCTTGC		GTTGGGCGTAATGTTGAC	
Hypothetical protein	ATCTTTGGATGGGTGGTTTG	142	ATTCGGAGAACACCCAGTA	154
	TGTTCTTCTTCGGTTTGAGG		AACAGCACCAGAAAGAACA	
Clathrin coat AP1	ATTCGCCCAAGTTTAAGACC	200	CATCACCTGGACGATCAA	191
	CGTGGTGAAGTACGGAATCT		GGTATCTCGAACTTGACCTG	
SGF11	CCGCATCAGTTTATTAGTCG	211	TCGACCAACAGCAAAAAG	129
	CAGCGAATCCAGCAGATAGT		ACTAGCAATTCGAGACGAGT	
Casein Kinase II	CTTATTTTGCGGTCATCGTT	186	AAAGTGCGTCGTGAAGAT	102
	TGTTTTCCCCTGGTTATTCG		AGCGTAATAATGTTGGTTCC	
PAF/WD repeat protein	ACGGATGATGGAACACATTT	196	CTAAGATTCACGGACGATG	130
S7 ribosomal protein	AAATAAATTCGCTATGGTTTTCGG	182	AAATAAATTCGCTATGGTTTTCGG	182
	CCTTCTTGCTGTTGAACTCG		CCTTCTTGCTGTTGAACTCG	

3.2.3 Isolation of the Resistance Factor and Susceptibility Factor Genes in Wild-Caught *Aedes vexans* and *Coquellettidia perturbans* mosquitoes

Mosquitoes were collected throughout the city of Winnipeg MB using CDC Mosquito Light Traps (also known as New Jersey Light Traps) by staff of the City of Winnipeg Insect Control Branch. These traps can vary in their efficiency to attract different species, but are used routinely to monitor pest mosquitoes in Winnipeg (Winnipeg Public Works 2015; Reinert 1989). These traps often are more attractive to females (Ellis 2001), and in the traps, only females were obtained for further analysis in this study. RNA was extracted from 10 adult female *Coquellettidia perturbans* and 10 adult female *Aedes vexans* mosquitoes using a GeneJET RNA Purification Kit (Fermentas/Thermo Scientific) according to the manufacturer's instructions and as described in section 3.2.2. RNA was then DNase-treated for cDNA synthesis as described in section 2.2.1.

Degenerate primers were designed for the clathrin coat adaptor protein AP1 susceptibility factor and clathrin coat adaptor AP3 resistance factor genes identified in Cheng *et al.* 2010 along with a ribosomal protein gene S7 (Table 3.3) by creating alignments of known sequences of the genes in *Aedes, Anopheles,* and *Culex* mosquito species using Geneious software and Sigma-Aldrich's OligoEvaluator^{TM}. The degenerate primers were used in a 50 µl polymerase chain reaction (PCR) reaction (Econotaq, Lucigen) using *A. vexans* and *C. perturbans* cDNA as templates. Reverse touchdown PCR was performed using annealing temperatures starting at 8°C below the primer melting temperatures and increasing by 0.2°C for 40 cycles to ensure that during the first cycle, primers bound with low stringency to the desired gene sequence, and in subsequent cycles, the primers bound with higher stringency to gene sequences. The PCR reactions were resolved on a 1% agarose gel supplemented with ethidium bromide and visualized using a bio imaging system (Syngene). Amplicons were excised from the gel and purified with

Thermo Scientific's GeneJet Gel Extraction Kit according to manufacturer's instructions. Gel-purified samples were ligated into the pJet1 cloning vector (Thermo Fisher Scientific; Figure 2.3), and the ligated plasmids were transformed into Subcloning Efficiency^{TM} DH5α Chemically Competent *Escherichia coli* cells (Invitrogen) using a heat-shock method as described by the manufacturer. 200µL of the cells were plated onto LB agar plates (1% bacto-tryptone 0.5% bacto-yeast extract, 1% NaCl, 1.5% bacto-agar) substituted with 50 mg/ml ampicillin and incubated at 37°C overnight.

Table 3.3. Degenerate primers used to amplify gene sequences in mosquito tissues.

Gene amplified	Primer sequence	Level of degeneracy	Size of fragment (nt)
Ribosomal Protein S7	AAATAAATTCGCTATGGTTTTCGG	0	184
	CCTTCTTGCTGTTGAACTCG	0	
Clathrin AP1	CAAGGCSAAGTCGCAGT	12	224
	GTAGTCBCCGTTCTGGGT	16	
Clathrin AP3	TRGATCTVATYTTCCACGC	2	370
	ATCRGGYAAYTTRATGTCCTT	3	

Transformed bacterial colonies were screened for PCR amplicon inserts using a PCR screening method. Bacterial colonies were streaked onto a master plate and subsequently dipped into PCR tubes containing a standard PCR reaction mixture and plasmid-specific primers described in section 2.2.1. The cells were lysed by incubating the PCR tubes at 95°C for 10 minutes prior to PCR amplification to release the plasmid DNA templates. An annealing temperature of 63.7°C was used during the PCR cycles. Samples were resolved on a 1.5% agarose gel substituted with ethidium bromide to identify colonies containing the appropriately-sized insert fragments. Bacterial colonies were grown in a shaking incubator (225 rpm, 37°C overnight) in 3 ml of LB broth substituted with 50 mg/ml ampicillin. The plasmid DNA was

isolated from the bacterial cells and purified using a GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific).

The identities of the sequences were analyzed by sequencing the amplicons (TCAG Sequencing Facility) and by comparing them to the genome databases available at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). A Basic Local Alignment Search Tool (BLAST) was used to compare sequenced fragments to known gene sequences, and gene alignments were performed using Geneious software to confirm the identities of the clathrin coat adaptor protein genes in both *A. vexans* and *C. perturbans*.

3.2.4 Expression Levels of Genes Affecting WNV in Whole Bodies of wild-caught *Aedes vexans* and *Coquellettidia perturbans* mosquitoes

Protein sequences of putative clathrin coat adaptor proteins from five mosquito species (obtained from GenBank) were aligned with ClustalX using default parameters (Larkin *et al.*, 2007). Maximum likelihood phylogenetic trees, using mosquito, other invertebrates, and vertebrate clathrin coat adaptor protein sequences were constructed using MEGA (6.06) (Appendix, supplementary Figure S3). The trees were tested using a bootstrap value of 1000 and the evolutionary distance of the phylogenetic tree was computed using a Poisson correction method in addition to eliminating gaps (Felsenstein 1985). The percent identities to *Aedes aegypti* were calculated using Geneious software (Appendix, supplementary Table S1).

Gene- and species-specific primers were designed for qRT-PCR gene expression analyses (Table 3.4) using the Integrated DNA Technologies (IDT) PrimerQuest program. Primers were designed to produce amplicons fewer than 200 bp and have annealing temperatures within 1°C of each other. The ribosomal protein S7 gene was used as an internal reference gene for comparing quantities of clathrin coat adaptor protein genes in both mosquito species.

Table 3.4. Gene-specific qRT-PCR primers designed from cloned and sequenced amplicons obtained using degenerate primer PCR.

	Gene	Primer Sequence	Size of Fragment (nt)
S7	***A. vexans* F**	TATCCTGGAGCTGGAGAT	87
	***A. vexans* R**	CTGTTGAACTCGATCTCAC	
	***C.perturbans* F**	ATAAATTCGCTATGGTTTTC	116
	***C.perturbans* R**	GAGATCGGAGTTCATTTC	
Clathrin AP3	***A. vexans* F**	CTGCCGCAACAGATAAAG	101
	***A. vexans* R**	GTGAGTAACGACGTATCAAAG	
	***C.perturbans* F**	GTTCGTGTTTGGTCAGAATTT	172
	***C.perturbans* R**	TCGAATCGAGGAACAGAATAAG	
Clathrin AP1	***A. vexans* F**	CTCCGATTCAGGTCAAGT	128
	***A. vexans* R**	GCCGTTCTGGGTAATGTA	
	***C.perturbans* F**	AGATGTAGTCTCCGTTCTG	200
	***C.perturbans* R**	GCGGAAAGGAGTATCTGA	

qRT-PCR was used to determine the levels of expression of the clathrin coat adaptor proteins in both of *A. vexans* and *C. perturbans*. For each cDNA sample, qRT-PCR was performed using a CFX Connect^{TM} Real Time PCR Detection System as described in section 2.2.2 using annealing temperatures 5°C below the primer melting temperatures.

The relative amount of clathrin coat adaptor protein transcripts in the mosquito samples was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) where transcript levels are normalized to the internal standard (S7) using the equation described in section 2.2.2.

3.2.5 WNV Gene Expression in Wild-Caught *Aedes vexans* and *Coquellettidia perturbans* mosquitoes

RNA was extracted from 20 adult female *Coquellettidia perturbans* and 20 adult female *Aedes vexans* mosquitoes using a GeneJET RNA Purification Kit (Fermentas/Thermo Scientific) as described in section 3.2.2. RNA was then DNase-treated for cDNA synthesis as described in section 2.2.1, and RT-PCR and gel electrophoresis was used to determine whether or not WNV was present in any of the individual mosquitoes as described in section 2.2.1.

3.2.6 Knockdown of Clathrin Coat Adaptor Protein Genes and WNV's Ability to Bind-to and Infect Mosquito Cells

3.2.6.1 Cloning into pJET

RNA was extracted from *A. aegypti* and *Cx. quinquefasciatus* adult whole bodies using a GeneJET RNA Purification kit (Thermo Scientific) according to the manufacturer's instructions. cDNA synthesis and RT-PCR were performed as described in section 2.2.1 using the RT-PCR primers listed in Table 3.5. Two 50 µL PCR reactions were performed as described in section 2.2.1 using the annealing temperature of 49°C.

Table 3.5. Primers used to amplify the clathrin coat adaptor protein gene sequences in mosquito tissues for subsequent cloning.

Gene amplified	Species	Primer sequence	Size of fragment (nt)
Clathrin AP1	*A.aegypti*	ATTCGCCCAAGTTTAAGACC	200
		CGTGGTGAAGTACGGAATCT	
	Cx. quinquefasciatus	CATCACCTGGACGATCAA	130
		GGTATCTCGAACTTGACCTG	
Clathrin AP3	*A.aegypti*	ATTCGCCCAAGTTTAAGACC	405
		GCTATCTTGGTGAGTAACG	
	Cx. quinquefasciatus	CAGTATTTTAACGAAGACATG	488
		GGAAGATCAGGTAATTTGA	

The PCR reactions were pooled together and were resolved on a 1.5% agarose gel supplemented with ethidium bromide, and the fragments were excise from the gel and purified with a High-Speed Plasmid Mini Kit (Geneaid). Gel purified samples were ligated into the cloning vector pJET (Fig. 2.4) using a CloneJET PCR Cloning Kit (Thermo).

Subcloning Efficiency™ DH5α Chemically Competent *E. coli* cells (Invitrogen) were transformed with the ligated plasmids using a heat-shock method as described in section 2.2.1.

The cells were then plated on LB agar (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, 1.5% bacto-agar) plates with ampicillin (50 mg/ml).

Transformed bacterial colonies were tested for the presence of PCR fragment inserts using a PCR colony screening method described in section 2.2.1. PCR reactions were resolved on a 1% agarose gel to identify any colonies containing plasmids with appropriate-sized inserts. The bacterial colonies were then grown in 3 ml of LB broth with ampicillin (50 mg/ml) overnight at 37°C with shaking at 225 rpm to give adequate aeration to the cells. The plasmid DNA was purified from the bacteria using Geneaid's High-Speed Plasmid Mini Kit.

3.2.6.2 Sub-cloning *A. aegypti* and *Cx. quinquefasciatus* clathrin coat adaptor proteins AP1 and AP3 for dsRNA delivery

The clathrin coat adaptor protein AP1 and AP3 gene fragments were excised from the pJET plasmid using the restriction enzymes *XbaI* and *XhoI* and then ligated to the dual T7 vector pL4440 (Fig. 3.2), kindly provided as a gift from Andrew Fire (Addgene plasmid #1654), using T4 ligase (Invitrogen). *In vitro* transcription of double-stranded RNA (dsRNA) is facilitated by this plasmid by the convergent T7 promoters it possesses. The ligated plasmids were used to transform DH5α cells as described in section 2.2.1 and the bacterial colonies were PCR screened using the pL4440-specific primers: pL4440Fwd: 5'ACCTGGCTTATCGAA and pL4440Rev: 5' TAAAACGACGGCCAGT with an annealing temperature of 57°C. Plasmids were purified from the bacterial cells as described in section 2.2.1 using Geneaid's High-Speed Plasmid Mini Kit, and samples were sent for sequencing at the TCAG sequencing facility. Sequence identities were confirmed using NCBI's BLAST program and gene alignments using Geneious software.

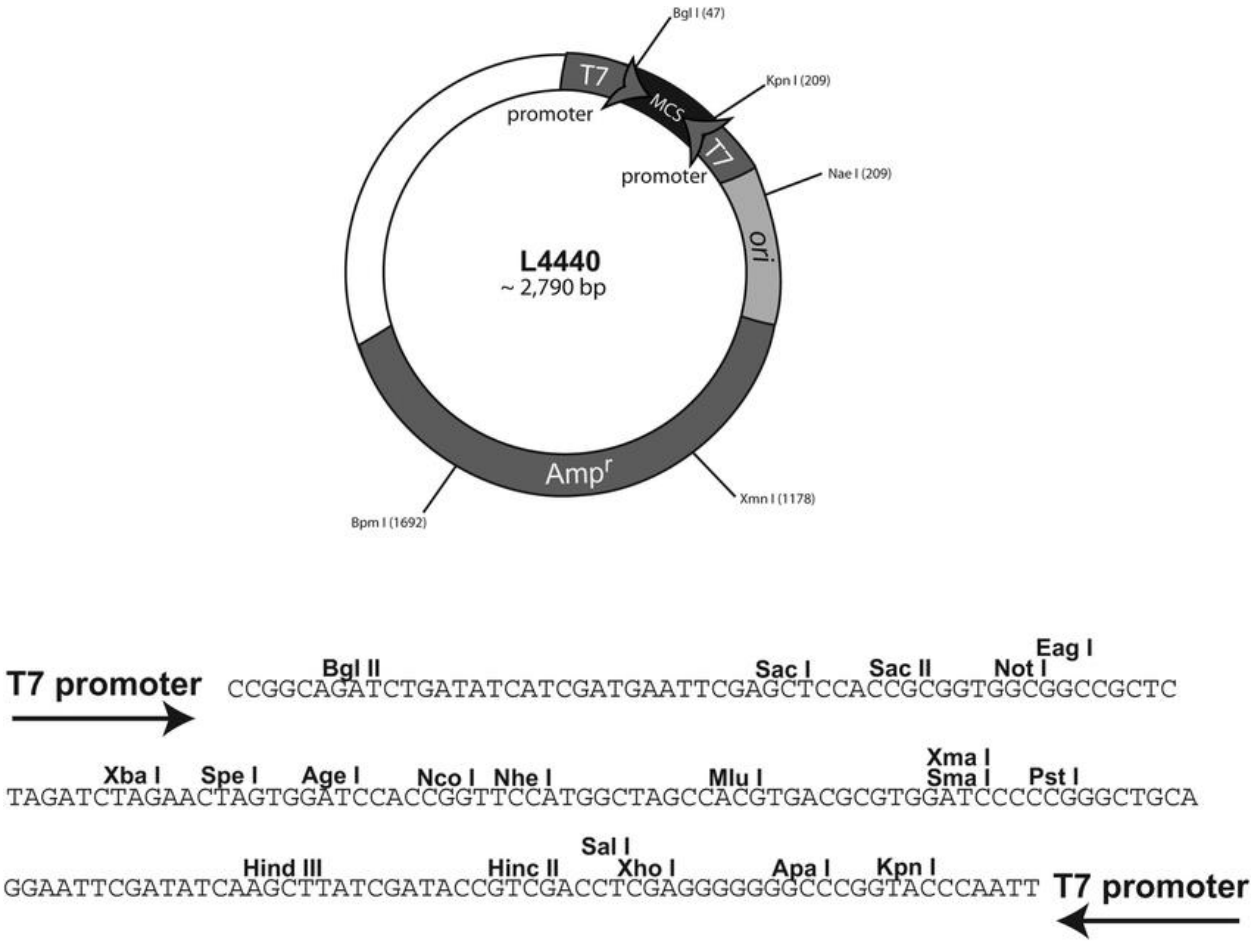


Figure 3.2. pL4440 vector map showing the dual T7 promoter sites and restriction enzyme sites. L4440 was a gift from Andrew Fire (Addgene plasmid #1654).

3.2.6.3 *In vitro* transcription of dsRNA

The clathrin coat adaptor protein gene fragments cloned into pL4440 were PCR-amplified along with the flanking convergent T7 promoter sequences from the dsRNA expression plasmid using the specially designed pL4440 primers listed above in section 3.2.6.2 in order to obtain sufficient DNA template for *in vitro* transcription from each of the pL4440 plasmids. Two standard 50 µL PCR reactions were performed for each of the two genes in each of the two mosquito species using the pL4440 plasmid-specific primers described in section 3.2.6.2 using an annealing temperature of 57°C. Gene fragments were resolved on a 1% agarose

gel and the bands were excised. Following excision from the gel, the two 50 µl PCR reactions were pooled together to increase the concentration of template in each reaction and purified using Thermo Fisher's GeneJET gel extraction kit according to the manufacturers specifications.

The gel-extracted PCR templates (1 µg) were used in 20 µl *in vitro* transcription reactions with the MEGAscript® RNAi kit (Ambion) according to the manufacturer's specifications to produce concentrated dsRNA complementary to each gene of interest (clathrin coat adaptor proteins AP1 and AP3). By incubating the purified template with NTPs, buffer, water, and T7 RNA polymerase, this kit synthesizes complementary single-stranded RNA that hybridizes to form dsRNA by first denaturing the strands at 75°C and slowly cooling the reaction to room temperature. The dsRNA was then treated with DNase and RNase to remove the template DNA as well as any ssRNA. The purified dsRNA was resolved on a 1.5% agarose gel to confirm the fragment size and sample purity, and the concentration was estimated using a NanoVue Spectrophotometer (GE Healthcare). A negative control dsRNA, specific to the β-glucuronidase (*gus*) gene of *E. coli*, was kindly provided by Dr. Steven Whyard to be used as a control.

3.2.6.4 Maintenance of *A. aegypti* and *Cx. quinquefasciatus* cell lines

CCL-125 cells (ATCC), an *A. aegypti* cell line derived from mosquito larvae, were maintained in MEM/EBSS media (Thermo Fisher Scientific) supplemented with 20% (V/V) heat-inactivated fetal bovine serum (HI-FBS, Thermo Fisher Scientific). *Culex quinquefasciatus* cells (Hsu) established from embryonic cells were kindly donated from Robert Tesh (University of Texas Medical Branch) and were maintained in L-15 media (Thermo Fisher Scientific) supplemented with 15% HI-FBS (Thermo Scientific) and 10% tryptose phosphate broth

(Teknova). Cells were grown in T-75 flasks (BD Falcon) at 28°C, 5% CO_2, at 80% relative humidity and were split 1:4 weekly.

3.2.6.5 Transfection of *A. aegypti* and *Cx. quinquefasciatus* cell lines

Cells (2 ml CCL-125 cells; 2 ml Hsu cells) were seeded into six-well plates (BD Falcon) prior to transfection with dsRNA complementary to the clathrin coat adaptor protein genes AP1 and AP3. When cells reached 50-80% confluence, the media was withdrawn from the wells and the cells were washed with 2 ml of 10X PBS (Thermo Fisher Scientific) before adding 2 ml of MEM supplemented with 10% (V/V) HI-FBS for CCL-125 cells and 2 ml of L-15 supplemented with 7.5% (V/V) HI-FBS and 5% (V/V) tryptose phosphate broth for *Culex* cells. Cells were then transfected using Lipofectamine® RNAiMAX and Opti-MEM media (Invitrogen) and the appropriate amount of dsRNA according to the manufacturer's instructions and protocol optimization. To evaluate knock-down of gene expression of the gene of interest, dosages of 1, 5, 10, and 20 pmol of the appropriate dsRNA were administered to cells and qRT-PCR was performed to evaluate levels of knockdown. Briefly, for CCL-125 cells, the appropriate amount of dsRNA (10 pmol for AP3 and the control β-glucuronidase (gus) genes; 20 pmol for the AP1 gene) was added to Opti-MEM media to a final volume of 150 µl. In a separate 1.5 ml microcentrifuge tube, 9 µl of Lipofectamine® was added to 141 µl of Opti-MEM media, mixed gently, and incubated at room temperature for five minutes. The two solutions were combined, mixed gently, and incubated at room temperature for 5 minutes. 250µL of the mixture was administered to the cells. For *Culex* cells, 10 pmol each of AP3, AP1, and gus dsRNA was added to Opti-MEM media to a final volume of 75 µl. In a separate 1.5 ml microcentrifuge tube, 4.5 µl of Lipofectamine® was added to 70.5 µl of Opti-MEM media, mixed gently, and incubated at room temperature for five minutes. The same protocol as that for CCL-125 cells was used before

administering 100µL of the mixture to the cells. Three independent replicates of this experiment were conducted.

The cells were incubated with the transfection mixture for 48 hours in a 5% CO_2, humidified incubator at 28°C. Total RNA was subsequently extracted from each well using Thermo Scientific's GeneJET RNA Purification Kit as described in section 2.2.1 for use in qRT-PCR. The tissue homogenization step using a plastic pestle was omitted. cDNA synthesis was prepared using a qScript SuperMix cDNA synthesis kit (Quanta BioSciences) as described in section 2.2.1. Primers used in RT-PCR for cDNA verification are listed in Table 3.2, and fragments were resolved on a 1.5% agarose gel supplemented with ethidium bromide, as described previously. Levels of knock-down of gene expression of the gene of interest were evaluated using qRT-PCR as described previously using primers listed in Table 3.2. Expression levels of the clathrin coat adaptor protein genes relative to the S7 ribosomal control gene were evaluated separately in cells treated with dsRNA specific to the gene of interest as well as in cells treated with *gus* dsRNA, the negative control dsRNA. A comparison of knock-down of gene expression was made between cells treated with the appropriate clathrin coat adaptor protein dsRNA and cells treated with *gus* dsRNA using the $2^{-\Delta\Delta CT}$ method.

3.2.6.6 Assessment of WNV infection of mosquito cells

CCL-125 cells (ATCC), and *C. quinquefasciatus* (Hsu) cell lines were maintained in media as described previously (section 3.2.6.4) and were sub-cultured in six-well plates for subsequent transfections and infections. Infections were performed by Cass Erdelyan at the National Microbiology Laboratory (Winnipeg, Canada). Vero cells (African green monkey kidney cells, ATCC) were maintained at 37°C in DMEM (GIBCO) containing 10% inactivated

FBS. West Nile virus (strain NY99) used in this study was kindly provided by Dr. Michael Drebot (National Microbiology Laboratory, Winnipeg, Canada) and the virus was propagated in Vero cells until used for mosquito cell infections.

The following procedures were performed by Cass Erdelyan at the National Microbiology Laboratory (Winnipeg, Canada). At approximately 80-100% confluence, CCL-125 and *Culex quinquefasciatus* cells were inoculated with WNV at a multiplicity of infection (M.O.I.) of 10, with the virus diluted in BA-1 medium (0.05M Tris buffer (pH 7.6), 4.75 mL Sodium bicarbonate (7.5%), Bovine Serum Albumin (1%), penicillin/streptomycin (100 units), and medium 199 with Earl's salts (up to 100 ml)). Unlike Vero cells, the mosquito cells showed no cytopathic effects, even 9 days post-infection.

3.2.6.7 Assessment of the effects of the dsRNAs on WNV infections in mosquito cells

Cells were transfected with the appropriate concentration and type of dsRNA 48 hours prior to infection with WNV as described above. Additional control treatments included cells without any transfection reagents. Cells were then challenged with 10 M.O.I. of virus and incubated for 48 h. The number of virions within the cell lysate as well as those released in the supernatant was measured using qRT-PCR as described above. Five independent replicates of this experiment were conducted.

3.2.6.8 Statistical Analyses

Statistical analyses were performed using GraphPad InStat software. Statistical significance of the data was assessed by performing unpaired t tests with a Welch correction.

3.3 Results

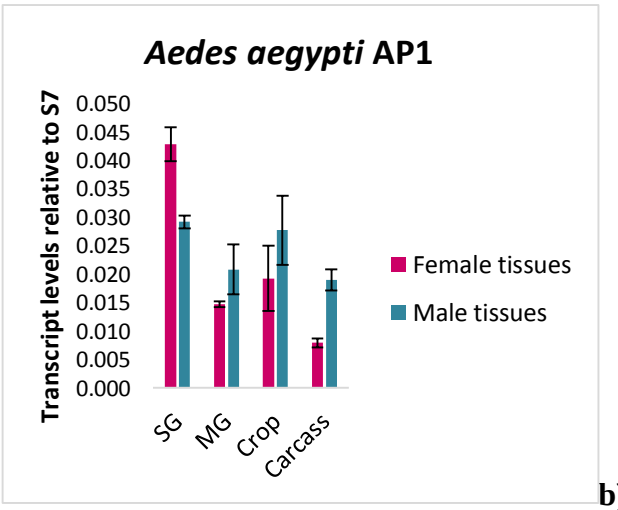
3.3.1 Expression Levels of Genes Affecting WNV in the Salivary Glands, Crop, Midgut, and Carcass of *Aedes aegypti* and *Culex quinquefasciatus*

Cheng *et al*. (2010) identified 13 genes that increased or decreased WNV levels in *A. aegypti* mosquitoes; however their study focused solely on the effects of two of those genes on WNV infection in mosquitoes. Because WNV is able to infect multiple different vertebrate and invertebrate hosts, it is likely that multiple factors influence WNV infection in different species and no single protein or molecule is solely responsible for regulating infection in any given host. For this reason, I performed qRT-PCR on six of the resistance factor genes and four of the susceptibility factor genes to determine their expression levels in different tissues of *A. aegypti* male and female mosquitoes (Appendix, supplemental Figure S4). Due to time constraints, only one resistance factor gene and one susceptibility factor gene was utilized for follow-up studies. The clathrin coat adaptor proteins AP1 and AP3 were chosen because interestingly, although they have similar roles in the cell, Cheng *et al*. (2010) identified one as a resistance factor (AP3 and the other as a susceptibility factor (AP1).

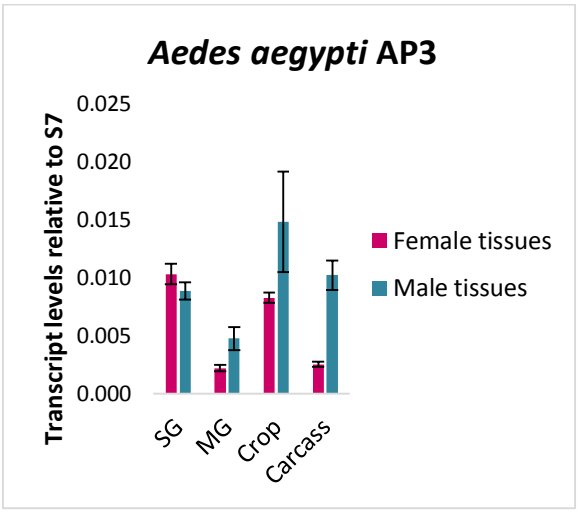
Phylogenetic analyses confirmed the identities of the clathrin coat adaptor proteins AP1 and AP3 in *A. aegypti* and *Cx. quinquefasciatus* mosquitoes, and interestingly, although gene sequences differed slightly, protein sequences of both AP1 and AP3 were identical in both species (Appendix, supplemental Table S1). Protein sequences of AP1 and AP3 were, however, significantly different from one another in both *A. aegypti* and *Cx quinquefasciatus*, with pairwise identities of 11.8% and 15.7% respectively. Quantitative RT-PCR was used to determine the level of gene expression of the clathrin coat adaptor proteins AP1 and AP3 in dissected salivary gland, midgut, crop, and carcass tissues in both male and female *A. aegypti* and *Cx. pipiens*. Within *A. aegypti* female tissues, AP3 gene expression was significantly higher

in salivary gland and crop tissues relative to both the midgut and carcass tissues (P<0.001) (Figure 3.3a). AP1 gene expression was significantly higher in salivary gland tissues than all other female tissues (P<0.005) and was significantly higher in the midgut relative to the rest of the carcass (P<0.0001) (Figure 3.3b). Within male tissues of *A. aegypti*, AP3 gene expression was significantly higher in salivary gland and carcass tissues relative to the midgut (P<0.0049) (Figure 3.3a), and AP1 gene expression was significantly higher in the salivary gland tissues relative to the rest of the carcass (P=0.0003) (Figure 3.3b). When comparing female and male *A. aegypti* tissues, both AP1 and AP3 gene expression was significantly higher in male midgut and carcass tissues relative to female midgut and carcass tissues (P<0.03) (Figure 3.3a). AP1 gene expression was significantly higher in female salivary gland tissues relative to male salivary gland, midgut, crop, and carcass tissues (P<0.007) and was also significantly higher in male carcass tissues relative to female carcass tissues (P<0.0001) (Figure 3.3b).

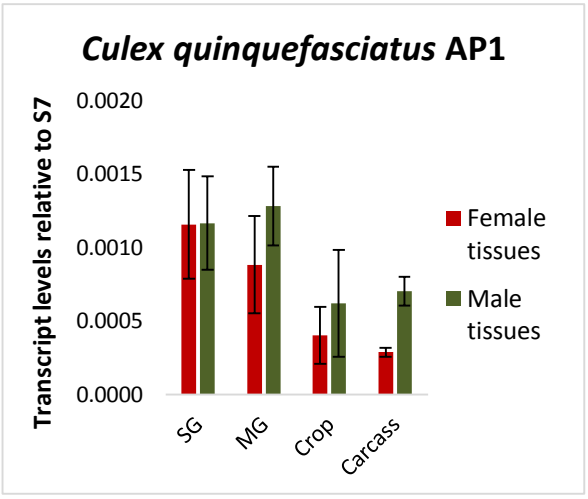
a)



b)



c)



d)

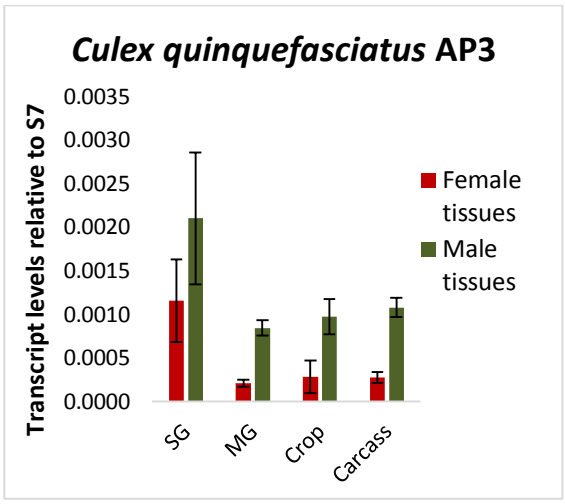


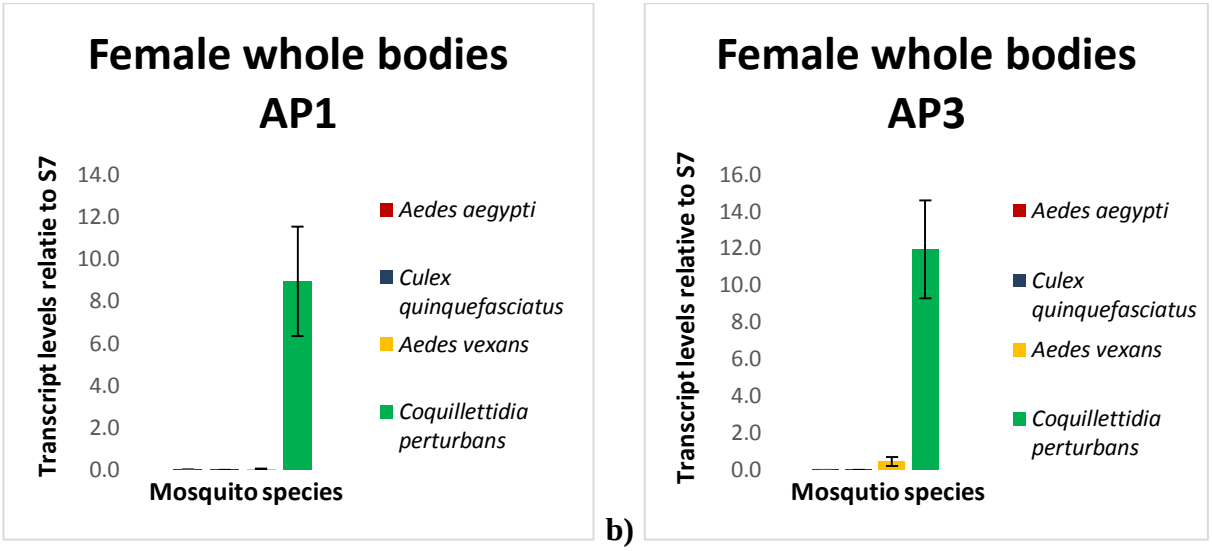
Figure 3.3. Gene expression analyses of the clathrin coat adaptor protein AP3 and AP1 genes in dissected tissues of a), b) *Aedes aegypti* and c), d) *Culex quinquefasciatus* males and females. Note the scales of the Y axes differ in each graph. SG = salivary gland; MG = midgut.

Within both *Cx. quinquefasciatus* males and females, there was no significant difference in AP1 and AP3 gene expression among all four of the dissected tissues (Figure 3.3 c,d). When comparing males and females, however, AP3 gene expression was significantly higher in male midgut, crop, and carcass tissues relative to female tissues ($P<0.03$) (Figure 3.3c), and AP1 gene expression was significantly higher in male carcass tissues relative to female carcass tissues ($P<0.05$) (Figure 3.3d). When comparing *A. aegypti* to *Cx. quinquefasciatus*, AP1 and AP3 gene expression was significantly lower in all four tissues of both male and female *Cx. quinquefasciatus* relative to *A. aegypti* ($P<0.05$) (Figure 3.3).

3.3.2 Expression Levels of Genes Affecting WNV in Whole Bodies of *Aedes vexans* and *Coquellettidia perturbans*

Phylogenetic analyses along with nucleotide and predicted protein alignments confirmed that the target sequences of the clathrin coat adaptor protein AP1 and AP3 genes were obtained from both *Aedes vexans* and *Coquillettidia perturbans* (Appendix, supplementary Figure S3).

Quantitative RT-PCR was used to determine the level of gene expression of the clathrin coat adaptor proteins AP1 and AP3 in whole bodies of wild-caught female *C. perturbans* and *A. vexans* mosquitoes. Ct values for the ribosomal protein S7 were within the conventional acceptable range (between 23 and 35) and were similar in all four mosquito species using the same amount of RNA from similar tissues, thus the S7 gene was an effective reference for qRT-PCR. Gene expression of both of the clathrin coat adaptor protein AP3 and AP1 genes was significantly higher in *C. perturbans* relative to *A. vexans* (P= 0.0005 and 0.018 respectively) (Figure 3.4). Interestingly, gene expression of both of the clathrin coat adaptor proteins was also significantly higher in *C. perturbans* relative to both *A. aegypti* and *Cx. quinquefasciatus* female whole bodies (P<0.018) (Figure 3.4).



a) **b)**

Figure 3.4. Gene expression of the clathrin coat adaptor proteins a) AP1 and b) AP3 in *C. perturbans, A. vexans, A. aegypti*, and *Cx. quinquefasciatus* female whole bodies.

RT-PCR determined that 0 out of 20 of the wild-caught female *A. vexans* and *C. perturbans* showed any gene expression of WNV NS2A, indicating that none of the mosquitoes were infected with WNV.

3.3.3 Impact of RNAi-mediated Knockdown of AP Genes on WNV's Ability to Bind-to and Infect Mosquito Cells

qRT-PCR analysis of untreated cell cultures revealed that transcript levels of both AP1 an AP3 were not significantly different *Cx. quinquefasciatus* cells (P >0.05); however higher levels of AP1 transcripts relative to AP3 were discovered in *A. aegypti* cells (P=0.0007; Figure 3.5). No significant differences were detected in either AP1 or AP3 transcript levels between *A. aegypti* and *Cx. quinquefasciatus* cell cultures. In dsRNA-treated *A. aegypti* cell cultures, qRT-PCR analysis showed that gene expression of the clathrin coat adaptor protein AP3 and AP1 were knocked down 76.5% and 100% respectively, and in *Cx. quinquefasciatus* cells, AP3 and AP1 showed 84.3% and 96.3% knockdown respectively (Figure 3.6).

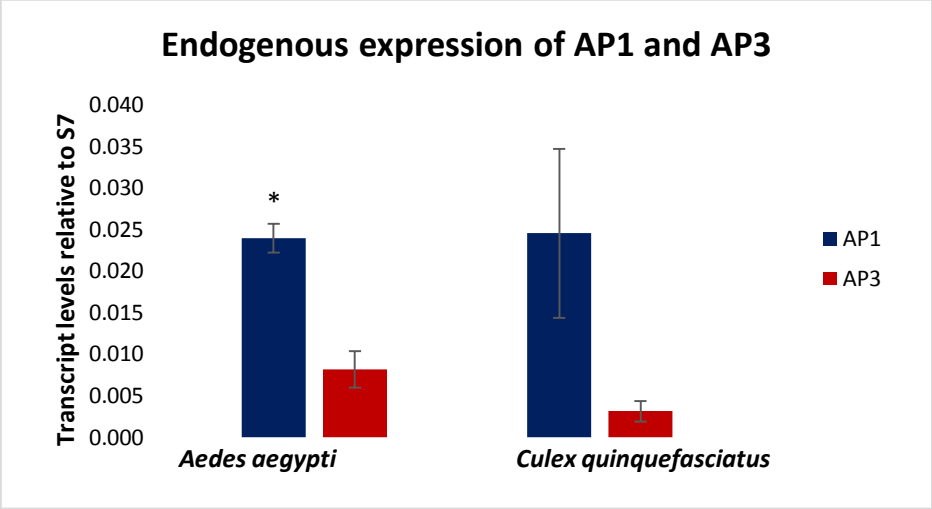


Figure 3.5. Endogenous gene expression of the clathrin coat adaptor proteins AP1 and AP3 in *Aedes aegypti* and *Culex quinquefasciatus* cell cultures prior to dsRNA treatments.

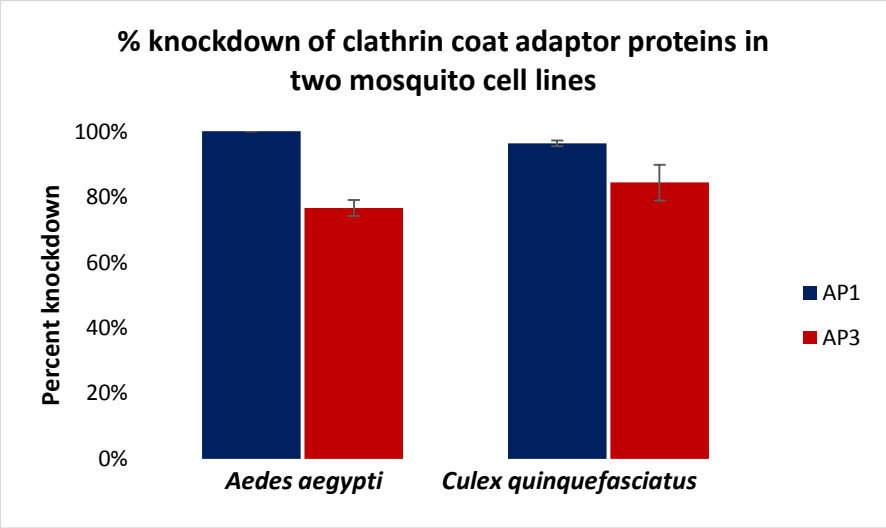


Figure 3.6. Confirmation of gene expression knockdown of the clathrin coat adaptor protein genes AP1 and AP3 in *A. aegypti* and *Cx. quinquefasciatus* cell cultures.

Cells were challenged with WNV to determine whether knockdown of the clathrin coat adaptor protein genes AP1 and AP3 affected WNV infection or viral shedding. In *A. aegypti* cells, knockdown of both AP1 and AP3 significantly increased (160% and 70%, respectively) the level of WNV transcripts in the cell lysate (P=0.02 and 0.05 respectively), whereas in *Cx. quinquefasciatus* cells, knockdown of both of the clathrin coat adaptor protein genes significantly decreased (85 and 90%, respectively) WNV transcripts in the cell lysate (P=0.05 and 0.03 respectively) (Figure 3.7). Additionally, in *A. aegypti* cells, WNV transcript levels were significantly higher in cells treated with AP1-specific dsRNA relative to cells treated with AP3-specific dsRNA (P=0.03). In the cell culture medium collected from the *A. aegypti* cell cultures, there was no significant difference in WNV transcripts detected among the gus, AP1, or AP3 dsRNA-treated cells, which suggests that there was no difference in the amount of virus shed from the cells in the various treatments (Figure 3.8). In the cell culture medium of *Cx. quinquefasciatus* cell cultures, WNV transcript levels were significantly lower in AP3 dsRNA-

treated cells relative to gus dsRNA-treated cells (P=0.018) but WNV transcript levels were not significantly higher in AP1 dsRNA-treated cells relative to gus or AP3 dsRNA-treated cells (Figure 3.8).

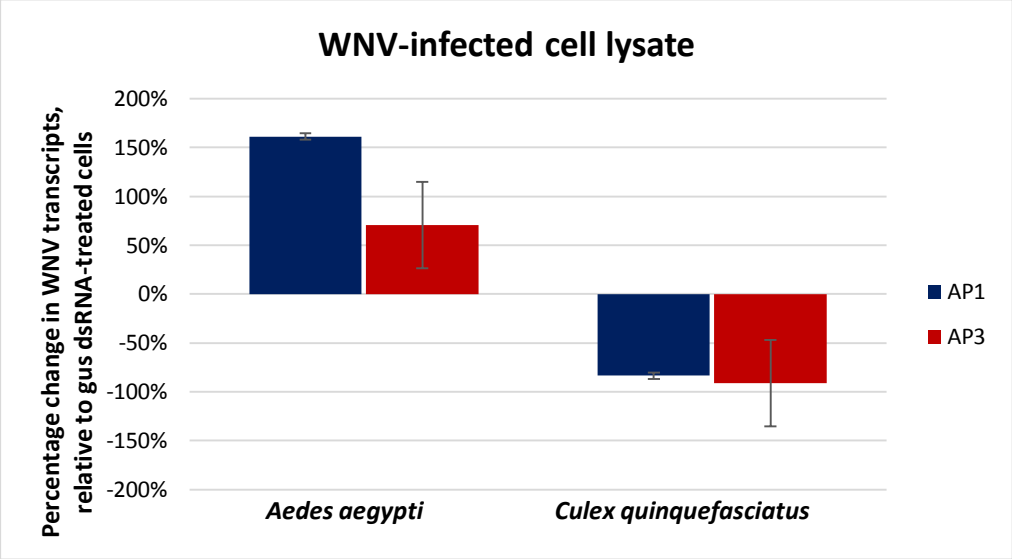


Figure 3.7. WNV transcript levels in *A. aegypti* and *Cx. quinquefasciatus* cell lysates following knockdown of the clathrin coat adaptor protein genes AP3 and AP1. Values represent the means and standard errors of five replicate experiments.

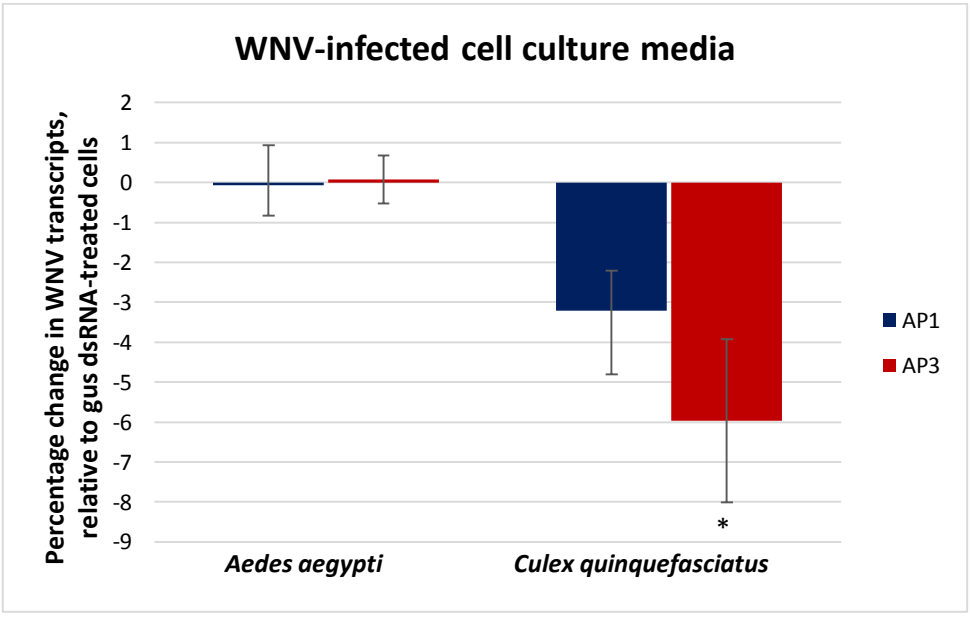


Figure 3.8. WNV transcript levels in *A. aegypti* and *Cx. quinquefasciatus* cell culture media following knockdown of the clathrin coat adaptor protein genes AP3 and AP1. Values represent the means and standard errors of five replicate experiments.

3.4 Discussion

Although mosquitoes are the only known vectors of WNV, certain species are more capable of transmitting the virus to different hosts (Turell *et al.* 2005 *et al.*; Turell *et al.* 2000). The reasons for these species differences in vectoring capabilities are not well defined; however feeding habits and host preferences likely play a role in the spread of the virus. Additionally, physiological differences among different mosquito vectors, such as the ability of the virus to quickly replicate to high levels, cross the midgut epithelial cells, and migrate to the salivary glands, as well as innate immune responses to WNV are also likely to influence vector competence (Blair 2011; Cheng *et al.* 2010; Turell *et al.* 2005; Sardelis *et al.* 2001; Turell *et al.* 2000). At the cellular and molecular levels, the number and/or type of host cell-surface receptor molecules present in key tissues may allow increased attachment of WNV to the cell membrane. Additionally, molecules involved in viral uptake through endocytosis, viral replication, and subsequent release of mature virions may be present in different concentrations or distributions in key tissues of different mosquito species, thus enhancing or inhibiting viral replication within different vectors. By examining gene expression of certain key proteins involved in the process of viral uptake into and release from host cells, we can gain a better understanding of how WNV is able to infect mosquito host cells more readily in certain species relative to others (Turell *et al.* 2005; Turell *et al.* 2000), thereby allowing them to act as more competent vectors.

The clathrin coat adaptor proteins AP1 and AP3 are both present in coated vesicles and aid in transport of proteins from inside endosomes, and therefore are likely involved to some degree in the WNV replication cycle within mosquito cells (Vassilieva and Nusrat 2008; Popova *et al.* 2013; McPherson *et al.* 2000). Cheng *et al.* (2010) described AP1 as a susceptibility factor and AP3 as a resistance factor in *A. aegypti*, where their natural functions were believed to

increase or decrease viral load respectively following infection with WNV. At the protein level, AP1 and AP3 are significantly different, and therefore their functions in the cell may be dissimilar even though they are both involved in the endocytic pathway and vesicle trafficking within the cell. Interestingly, in contrast to Cheng *et al.* (2010), when gene expression of both AP1 and AP3 were knocked down in the *A. aegypti* cell line CCL125, derived from larval tissue, in this study, the level of WNV significantly increased in the cell lysate, which suggests that both of these clathrin coat adaptor proteins were acting as resistance factors in this species (Figure 3.7). Additionally, in these cultured *A. aegypti* cells, AP1 may confer even more resistance to WNV than AP3, because when knocked down, WNV levels were significantly higher than cells in which AP3 gene expression was knocked down. Also, since endogenous expression of AP1 was significantly higher than AP3 in *A. aegypti* cells (Figure 3.5), by achieving 100% knockdown of AP1 (Figure 3.6), the effects of reduction of gene expression were presumably more drastic than if endogenous expression was relatively low to begin with. Conversely, in the *Cx. quinquefasciatus* cell line (Hsu) derived from ovarian tissue, endogenous expression of both AP1 and AP3 were not significantly different, and when both of the clathrin coat adaptor proteins were knocked down, WNV levels in the cell lysate decreased significantly (Figure 3.7), indicating that in this species, both AP1 and AP3 may be acting as susceptibility factors. Curiously, in *A. aegypti* cells, there was no significant difference detected in the amount of WNV transcripts in the cell culture medium of cells treated with AP1, AP3, and the β-glucuronidase (gus) control dsRNA, and because qRT-PCR has been demonstrated to be a suitable indirect method of detecting virions in organisms including mosquitoes (Faye *et al.* 2013; McCausland & Crotty 2008; Watzinger *et al.* 2006; Ryan *et al.* 2004), these data suggest that the number of virions shed from the cells was similar in all three dsRNA treatments (Figure 3.8). It is, however,

possible that a reduction in the amount of AP proteins involved in vesicle trafficking could inhibit transport of mature virions to the cell surface, resulting in reduced viral shedding into the culture medium. In the *Culex* cell line, WNV transcript levels were significantly lower in the cell culture medium where AP3 was knocked down relative to gus dsRNA-treated cells, which suggests that less virions were shed from the cells (Figure 3.8). These data suggest that AP3 may aid in viral shedding from the cell, further implying that this clathrin coat adaptor protein is acting as a susceptibility factor in this species of mosquito, as increased amounts of WNV released from the cell allows other cells to become infected with the virus. The reduced amount of WNV present inside cells as well as virus shed into the cell culture media following knockdown of AP3 supports the theory that in the *Cx. quinquefasciatus* cell cultures, AP3 was acting as a susceptibility factor to WNV in this study. Similarly to what was found in *A. aegypti* cells, cells in which AP1 was knocked down displayed no difference in the amount of WNV shed into the medium relative to control cells, suggesting no apparent role of AP1 in the process of exocytosis of WNV virions (Figure 3.8).

While Cheng *et al.* (2010) argued that AP1 is a susceptibility factor and AP3 is a resistance factor in *A. aegypti*, my findings suggest that both clathrin adaptor proteins act as resistance factors in the cultured *A. aegypti* cells. The discrepancy in the findings of these two studies most likely reflect differences in the cell types examined. In the Cheng *et al.* (2010) study, intact mosquitoes were first injected with dsRNA followed 3 days later with virus into their body cavities, and subsequently viral infection loads were determined by qRT-PCR. In contrast, in my study, cultured cells were first transfected with dsRNAs, then infected with virus, before measuring viral load by qRT-PCR. By injecting intact insects, Cheng and colleagues did not examine whether there were differences in responses in different tissues or cell types, but

showed that in the adult mosquito overall, the clathrin adaptor proteins showed opposing roles in modulating WNV infections in the *Aedes* mosquitoes. It is worth noting, however, the injection of the mosquitoes with the virus into the abdomen is not the natural route of infection of WNV, and hence, Cheng and colleagues' study would not necessarily identify the role of the adaptor proteins in WNV infections in the most relevant of tissues (e.g. gut and salivary glands), although cell cultures used in my study also do not represent whole tissues or reflect infections in multiple cell types. It is possible that in different tissues, clathrin coat adaptor proteins may be differentially facilitating endocytosis or exocytosis, thereby acting as resistance factors, while in other tissues, they are acting as susceptibility factors. As a result, what Cheng *et al.* (2010) observed was a combined effect of the two opposing roles of both AP1 and AP3 throughout the whole body of *A. aegypti* mosquitoes. In my study, I was unable to perform virus injections into mosquitoes, due to lack of access to the NML, and relied exclusively on cell cultures for my analyses. These cell cultures also were suboptimal in assessing the most relevant cell types interacting with WNV. As the cells were derived from larval insects, they would not represent all cell types found within adults, and because not all cell types survive the culturing process (Rothblat & Cristofalo 1972), the cell culture assays do not fully reflect the cellular interactions that occur in normal mosquito WNV infections. Both *A. aegypti* and *Cx. quinquefasciatus* cell lines are adherent in nature and exhibit epithelial cell morphology (ATCC, HSU), and therefore may not represent all cell types that could be infected in the mosquito. It is therefore not surprising that different interactions were observed in the two studies, and although the genes targeted in my study may have different effects in cultured cells relative to cells in specific tissues *in vivo*, all eukaryotic cells require endocytosis as part of their normal function, whether they are in culture or in the body of an organism, so it is highly likely that RNAi targeting of the

clathrin coat adaptor protein genes in my study are relevant to examine the effects of WNV infection. If time permits, and access can be attained to the NML, it would be worthwhile performing injections as well as feeding virally-infected blood to both species of mosquitoes to make more valid comparisons, and to determine if Cheng *et al.*'s (2010) findings can be replicated.

To gain a better understanding of the dynamics of WNV infection inside these two species of mosquito cells and to further clarify the results obtained from these data, additional future experiments could include producing a standard curve to gain absolute values of the number of WNV transcripts both in cell lysates and the media as well as protein analyses to confirm gene expression analyses. Additionally, real-time fluorescence microscopy imaging could identify the pathway taken by WNV through the cells as well as elucidate the roles of clathrin coat adaptor proteins in the process of viral uptake through endocytosis as well as viral shedding through exocytosis.

When examining the clathrin coat adaptor proteins in adult mosquitoes, differences in gene expression was observed among four key tissues within both males and females. In *A. aegypti* females, both the AP1 and AP3 genes were expressed at significantly higher levels in salivary glands relative to all other tissues (Figure 3.3), and because both of those proteins act as resistance factors to WNV, it is possible that increased amounts of these proteins in the salivary glands could limit infection in these tissues, thus aiding in preventing the spread of WNV between hosts and reducing vector competence in this species. It is also likely that high expression of these proteins play a role in maintaining the normal function of salivary gland tissue because gene expression of both AP1 and AP3 is also high in males, even though the role of the salivary glands in males and females differ due to the different feeding habits. Protein

analyses would be needed to confirm that increased transcript levels in these tissues is, in fact, directly correlated with protein production. Additionally, increased AP1 expression in the midgut relative to the rest of the carcass could act as a first line of defense by limiting the ability of WNV to penetrate intestinal epithelial cells, thus preventing the distribution of the virus to the rest of the body. It is also possible that reducing the infection of epithelial cells in the gut tissue could diminish the likelihood of vertical transmission of the virus to the offspring of an infected female because oogenesis is related to female feeding habits. Oogenesis in mosquitoes only begins following the ingestion of a blood meal, and if the blood was contaminated with WNV, the virus could be passed on to the developing egg through the yolk which is derived from nutrients obtained from the blood meal (Briegel *et al.* 2003). If high levels of resistance factors are present in the gut epithelium, the amount of virus that would be able to be transferred to the eggs would be diminished. In males, the levels of clathrin adaptor proteins in different tissues has no bearing on WNV infection processes, as males do not take blood meals from hosts and therefore do not contribute to the spread of WNV by their feeding behaviour

When comparing male and female *A. aegypti* mosquitoes, both AP1 and AP3 gene expression was higher in male midgut and carcass tissue relative to the same tissues in females, potentially rendering females more susceptible to WNV than males because of the reduced amount of resistance factors present in those tissues. Additionally, differential levels of the two clathrin coat adaptor proteins found within females may relate to differences in physiology relative to males, and because of the differences found within midgut tissue, it is possible that AP1 and AP3 play a role in digestion, possibly relating to the blood-feeding habits of female mosquitoes. Higher gene expression of AP1 in female salivary gland tissue relative to all four male tissues indicates that the presence of resistance factors is high in the tissue that is most

likely to aid in the spread of WNV from one host to another through female feeding habits, and therefore if infection is prevented in this tissue, it is less likely for the virus to be transmitted to other hosts, resulting in lower vector competence.

Both of the clathrin coat adaptor proteins AP1 and AP3 were acting as susceptibility factors rather than resistance factors in *Cx. quinquefasciatus*, and although gene expression of both AP1 and AP3 was significantly lower in all four tissues in *Cx. quinquefasciatus* relative to *A. aegypti*, it is still possible that *Cx. quinquefasciatus* mosquitoes are more susceptible to WNV infection than *A. aegypti*. Within both males and females, no significant difference of both AP1 and AP3 gene expression was found among all four tissues, indicating that all tissues are equally susceptible to WNV; however higher expression of AP1 in male carcass tissue relative to females combined with higher expression of AP3 in male midgut, crop, and carcass tissue relative to females could indicate that males are more susceptible to WNV infection than females. As males may acquire WNV by vertical transmission of WNV from their mothers, it would be interesting to examine whether certain tissues within males are more prone to WNV infections as a result of differential distribution of key susceptibility proteins, such as the clathrin coat adaptor proteins. It will also be of interest to determine whether male to female transmission of the virus is possible, as this could provide a new avenue for the virus to be maintained within mosquito populations. Additionally, to date, no studies on WNV-related mortality in mosquitoes have been conducted; however it is possible that high levels of WNV in adult mosquitoes can lead to death, therefore limiting the spread of the virus. Analyses of pathological effects of WNV in adult mosquitoes would aid in determining if the virus can, in fact, lead to death in these organisms.

When examining gene expression of the clathrin coat adaptor proteins in female whole

bodies of two species of wild-caught mosquitoes, *A. vexans* and *C. perturbans*, higher levels of both AP1 and AP3 were found in *C. perturbans* relative to *A. vexans*, as well as to *A. aegypti* and *Cx. quinquefasciatus* female whole bodies (Figure 3.4). Because cell cultures for these species were unavailable during the time of experimentation, it was not possible to make similar comparisons of the roles of AP1 and AP3 as resistance factors or susceptibility factors in these species, however Sardelis *et al.* (2001) reported that vector competence of *C. perturbans* is low. If AP1 and AP3 are actually important factors in WNV infections in *C. perturbans*, they may both be acting as resistance factors in this species. Additionally, gene expression of both AP1 and AP3 was significantly higher in *A. vexans* relative to both *A. aegypti* and *Cx. quinquefasciatus* (Figure 3.4), and as Tiawsirisup *et al.* (2008) discovered, *A. vexans* is a competent vector of WNV. If AP1 and AP3 are relevant to WNV infection in this species, the relatively high levels of transcripts encoding these proteins may render this species more susceptible to the virus. Because none of the wild-caught mosquitoes possessed any detectible levels of WNV (results not shown), it is impossible to determine whether one species is more susceptible to WNV at this time. To further assess whether AP1 and AP3 are, in fact, acting as resistance factors in *C. perturbans* and susceptibility factors in *A. vexans*, experimental inoculation with WNV would need to be performed and viral loads within both of the species would need to be observed. Additionally, infected mosquitoes would need to be allowed to feed on hosts to determine the amount of virus transmitted to the hosts as well as the infection rates the virus caused following transmission.

Overall, vector competence of different mosquito species may be related to how WNV is able to infect and spread throughout different tissues within adult mosquitoes. If certain molecules or proteins are present in different concentrations in key tissues that confer resistance

or susceptibility to the virus, certain mosquito species may act as superior or inferior vectors to maintain or enhance the viral transmission cycle. By gaining an understanding of these key factors that contribute to vector competence, we will be better able to focus on controlling mosquito populations of species that play a key role in the transmission of WNV and may potentially reduce the risk of contracting the virus.

Chapter 4: Conclusions and Future Directions

The West Nile arbovirus poses a serious threat to public health due to the severity of symptoms that it can cause, including encephalitis and death, coupled with the lack of adequate forms of treatment and prevention currently available (Beckham & Tyler 2009; Kramer & Bernard 2001). Although a human vaccine is currently being developed for clinical trials (NIH 2015), no vaccine is accessible presently. Because of this, many avenues of research have been focused on understanding the dynamics and biology of WNV vectors and hosts in order to monitor outbreaks and improve mosquito control efforts (reviewed in Kramer *et al.* 2007; Hayes & Gubler 2006; Brinton 2002), with the aim of limiting the spread of the virus and impeding its transmission cycle. From the studies described in Chapter 2, it is evident that different bird species have differential infection rates and therefore it is possible that different species play more critical roles in maintaining the transmission cycle in animal populations. Similarly, as discussed in Chapter 3, roles of molecules putatively involved in WNV infection can vary across different mosquito species, which can affect their vector competence.

The high proportion of WNV-infected birds collected in this study made it difficult to draw many comparisons between infected and uninfected specimens; however, much higher levels of WNV in the heart, brain, kidney, and liver of crows relative to sparrow tissues suggest that this species is highly susceptible to the virus, as it can replicate to high titers throughout the body. These extensive systemic infections in crows likely account for the high mortality rate observed in this species (Lim *et al.* 2015; Yaremych *et al.* 2004), and why crows are often considered the sentinel species for WNV prevalence in a region (Lim *et al.* 2015; Julian *et al.* 2002; Edison *et al.* 2001). The high viral titers could define crows as important hosts in the WNV transmission cycle, although infected crows may die before many additional mosquitoes

can acquire a blood meal, and hence, these birds may not serve as a long-term viral reservoir in the environment. Because it was impossible to determine the timing of exposure to the virus to the time of death in the samples obtained for this study, I could not determine whether the differences in gene expression in the birds' tissues were directly correlated with the infection status of the bird. To clearly define how gene expression changes might be correlated with WNV infections, it would be more appropriate to establish lab-bred colonies of the two bird species that could be experimentally infected with WNV, and to examine at precise time points how the viral infection progresses and how gene expression changes due the course of infection. Furthermore, the route of viral infection from one tissue to the next could be investigated, which could help determine the cause of death from WNV in different bird species. In this study, high levels of WNV found in the heart tissue of crows relative to brain tissue could indicate that mortality from WNV infection in birds may be due to myocardial stress and/or heart failure, rather than neurological conditions, but until further experimentation is completed, this is still speculation.

In sparrows, the low levels of WNV found in the heart, brain, liver, and kidneys suggests that the virus may not replicate to high titers in these four tissues in this species. However, without knowing the duration of infection of these birds, it is not possible to make any definitive conclusion on this point. Sparrows have previously been defined as competent hosts for WNV transmission (Guerrero-Sánchez *et al.* 2011; Nemeth *et al.* 2009), which suggests that the low titers of virus in the locally-collected birds could be sufficient to maintain WNV in the region. Guerrero-Sánchez *et al.* (2011) demonstrated that viral titers above $10^{4.7}$ PFU/ml are sufficient to infect mosquito vectors, and that viral titers in sparrows reach or surpass those levels over the course of infection, rendering them competent amplifying hosts. It has also been demonstrated

that sparrows develop an immunity to WNV (Del Amo et al. 2014; Langevin et al. 2005), which may account for lower mortality rates in WNV-infected sparrows, and hence, they may be able to continuously infect multiple vectors throughout their lifespan, rendering them integral to the WNV transmission cycle. Again, experimental infection of lab-bred colonies would aid in comparing the relative host competency of the two bird species and in the underlying mechanisms that contribute to differential mortalities in the birds.

Data obtained from the studies described in in Chapter 2 revealed that although WNV levels were much higher in crows than in sparrows, expression of the putative WNV receptor, β3 integrin, was similar in both species and in all four tissues. Although it is possible that these data suggest that β3 integrin is not a primary regulator of WNV infection in birds, more follow-up is needed to further elucidate the role of this putative receptor in WNV infection. Because it was not possible to examine changes in integrin expression during the course of infection or to experimentally alter the levels of integrin and examine uptake of the virus, it is not possible to draw any significant conclusions regarding the role of integrins in WNV infection in different bird species. Additional analyses should include the examination of protein production to correlate to the gene expression data obtained in this study, as well as ligand binding assays to determine whether the WNV E glycoprotein does, in fact, bind to β3 integrin as a receptor molecule. Although there were limitations to this study, it has revealed differences in WNV infection and corporeal distribution between different bird hosts.

Similarly to bird hosts, different mosquito vectors also display differences in WNV infection (Turell et al. 2005; Turell et al. 2000). Data obtained from the studies conducted in Chapter 3 revealed that the clathrin coat adaptor proteins AP1 and AP3 played different roles in enhancing or inhibiting WNV infection in cell cultures derived from two different mosquito

species, *A. aegypti* and *Cx. quinquefasciatus*. In contrast to a study conducted by Cheng et al. (2010) that described AP1 as a susceptibility factor, enhancing WNV infection, and AP3 as a resistance factor, inhibiting WNV infection in *A. aegypti*, in this study, both AP1 and AP3 acted as resistance factors in *A. aegypti* cultured cells while in *Cx. quinquefasciatus* cultured cells, both of the adaptor proteins acted as susceptibility factors. The primary difference between my study and that of Cheng *et al.* (2010) is the type of models used to conduct the experiments. While Cheng et al. (2010) performed injections of dsRNA and WNV into adult mosquitoes, my study utilized cell cultures to assess the effects of RNAi-mediated knockdown of clathrin coat adaptor proteins on WNV infection. Neither study examined whether there are differences in responses in different tissues or cell types, nor did they infect tissues and cells through the natural route of infection, and although RNAi was effective in both *Aedes* and *Culex* cell cultures, it would be worthwhile to perform injections on adult mosquitoes and allow them to feed on infected blood to obtain more definitive, biologically relevant results. Mosquitoes could then be dissected and viral loads could be assessed in different tissues to determine whether adaptor proteins play more crucial roles in WNV infection in certain key tissues. Additionally, in addition to establishing standard curves to gain absolute values of the number of WNV transcripts, it would be beneficial to develop plaque assays to substantiate qRT-PCR results. Moreover, protein analyses of the clathrin coat adaptor proteins could confirm that knockdown of gene expression leads to a reduction of functional proteins, and therefore may be directly involved in mediating WNV infection in different species. Also, the roles of the adaptor proteins in the process of viral uptake could further be clarified by real-time fluorescence microscopy imaging, which could track WNV movements throughout the cell during the course of infection.

WNV is able to infect a wide variety of vertebrate and invertebrate hosts, and given the

range of different cell and tissue types it can infect, it is likely that multiple factors influence the virus's ability to bind, enter, and replicate within any given cell. Cheng *et al.* (2010) identified 13 genes that influenced WNV infection in *A. aegypti* mosquitoes but only focused on two for follow-up in their study. Likewise, in my study, only the two clathrin coat adaptor protein genes were studied; however resistance and susceptibility to WNV is likely the consequence of many factors that interrelate and although the clathrin coat adaptor proteins AP1 and AP3 appear to have a role in differential WNV infection rates, these two proteins are not working alone in dictating resistance or susceptibility to WNV. Future experiments that involve knocking down two or more resistance or susceptibility factors simultaneously would determine whether these genes provide an additive or synergistic effect on infection rates in different species.

Differences in vector competence of various mosquito species may be related to differences in physiology, where certain essential molecules are concentrated or distributed differently in certain key tissues. Moreover, it is possible that WNV resides predominantly in specific tissues during the course of infection. In this study, the clathrin coat adaptor proteins AP1 and AP3 were differentially expressed among salivary gland, midgut, crop, and carcass tissues, as well as between males and females in *A. aegypti* and *Cx. quinquefasciatus* mosquitoes. Because WNV is transmitted to hosts through infected saliva when female mosquitoes take a blood meal, levels of virus within the salivary glands are likely an important factor that influences the ability of a mosquito vector to transmit the virus, and therefore a potential indicator of vector competence. The clathrin coat adaptor proteins AP1 and AP3 act as resistance factors and susceptibility factors to either impede or enhance WNV infection, and in *A. aegypti*, both adaptor proteins acted as resistance factors in this study. Because transcript levels of both of these adaptor proteins were significantly higher in female salivary glands in this species, it is

possible that WNV infection in this tissue could be limited, thereby reducing the likelihood of transmitting the virus to subsequent hosts. Additionally, high transcript levels of AP1 in female midgut tissue relative to the carcass could aid in limiting the spread of WNV throughout the body, thereby reducing WNV infection in this species. Conversely, in *Cx. quinquefasciatus*, both of the adaptor proteins AP1 and AP3 acted as susceptibility factors in this study, and although gene expression was not significantly different among all four tissues, the role of these proteins suggest that WNV infection may be enhanced in this mosquito species, and therefore may aid in explaining why this species is known to be a competent vector of WNV (Turell et al. 2005; Sardelis et al. 2001). Although the roles of the adaptor proteins in *A. vexans* and *C. perturbans* were not determined in this study, previous studies discovered that vector competence of *C. perturbans* is low, while *A. vexans* is a competent vector (Tiawsirisup et al. 2008; Sardelis et al. 2001). Transcript levels of both AP1 and AP3 in adult female mosquitoes were significantly higher in both *C. perturbans* and *A. vexans* than *A. aegypti* or *Cx. quinquefasciatus*, and if these adaptor proteins are relevant to WNV infection in these species, they may be acting as resistance factors in *C. perturbans* and susceptibility factors in *A. vexans*. Possible mechanisms that might explain why certain molecules act as resistance factors may relate to the prevention of viral uptake into cells or inhibition of viral export, thereby preventing the spread to other cells and tissues. In contrast, susceptibility factors may be promoting viral uptake or enhancing export of the virus. In order to examine more completely whether the two clathrin coat adaptor proteins have opposing functions in different species, one would need to examine their normal functions in isolated cells, staining for their presence, and perhaps use endocytosis inhibitors to observe their altered locations. Additionally, it would be beneficial to track the import and export of certain molecules before and after RNAi-mediated knockdown of the two genes as it may reveal

differences in how the adaptor proteins impact WNV infection in different cell types as well as their level of importance in the process. Although there was no significant difference in endogenous expression of both AP1 and AP3 genes in *Cx. quinquefasciatus* cell cultures, in *A. aegypti* cell cultures, AP1 transcript levels were significantly higher than those of the AP3 gene. By administering dsRNA specific to AP1, 100% knockdown of AP1 gene expression was achieved, and in those cell cultures, WNV levels were significantly higher than in cell cultures where AP3 gene expression was reduced, suggesting that because endogenous expression of AP1 is high to begin with, a drop in transcript levels produces a larger impact on WNV infection in this species, and therefore AP1 may play a more important role. As stated previously, the WNV infection process is complex and likely involves many different molecules. Presumably, no single protein mediates resistance and susceptibility in a given species, and the role of the clathrin coat adaptor proteins cannot accurately be determined without manipulation of these insects to validate the experiments from this study. As a further form of verification, it would be beneficial to perform protein analyses to substantiate qRT-PCR data. Additionally, experimental infection and knockdown of the adaptor proteins in these species would further clarify the roles of these adaptor proteins in these mosquitoes. Vector competence could further be investigated by allowing infected mosquitoes to feed on hosts, and following knockdown of the clathrin coat adaptor protein genes, re-evaluate their abilities to transmit the virus to the hosts.

Understanding the mechanism by which WNV infects different hosts and vectors is an important step towards developing appropriate methods of prevention and treatment. By determining which receptors facilitate WNV attachment to cells as well as key proteins involved in the process of viral uptake into and release from host cells, a better understanding of the virus's ability to adapt to many vertebrate and invertebrate hosts will be achieved. This study has

provided insight into the distribution of WNV in different bird host tissues as well as the variations in infection levels in different bird species. Additionally, the roles of certain key molecules in vector competence of different mosquito species have been explored. By enhancing our knowledge of host susceptibility and vector competence, we will be better equipped to limit the spread of the virus and potentially reduce the likelihood of human infections.

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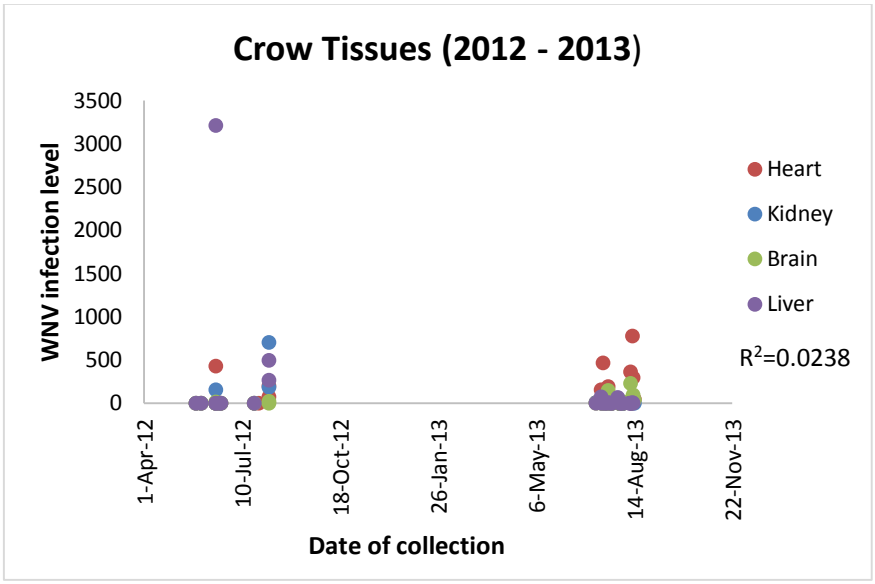
Appendix

a)



b)

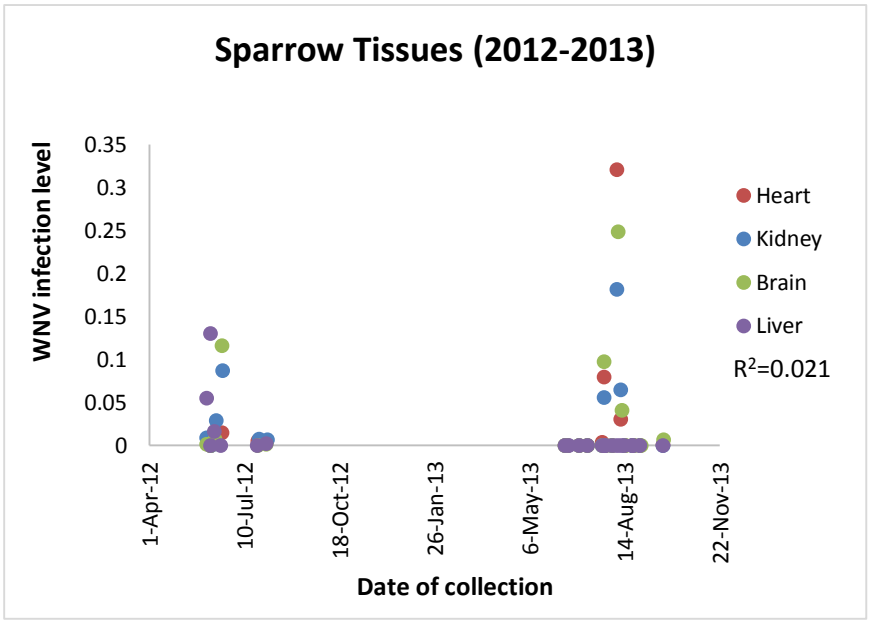


Figure S1. Correlation analysis between date of collection of dead birds and WNV expression levels in a) American crows and b) house sparrows. Note the difference in the y-axes scales on the two graphs, as crows have much higher levels of WNV transcripts than sparrows.

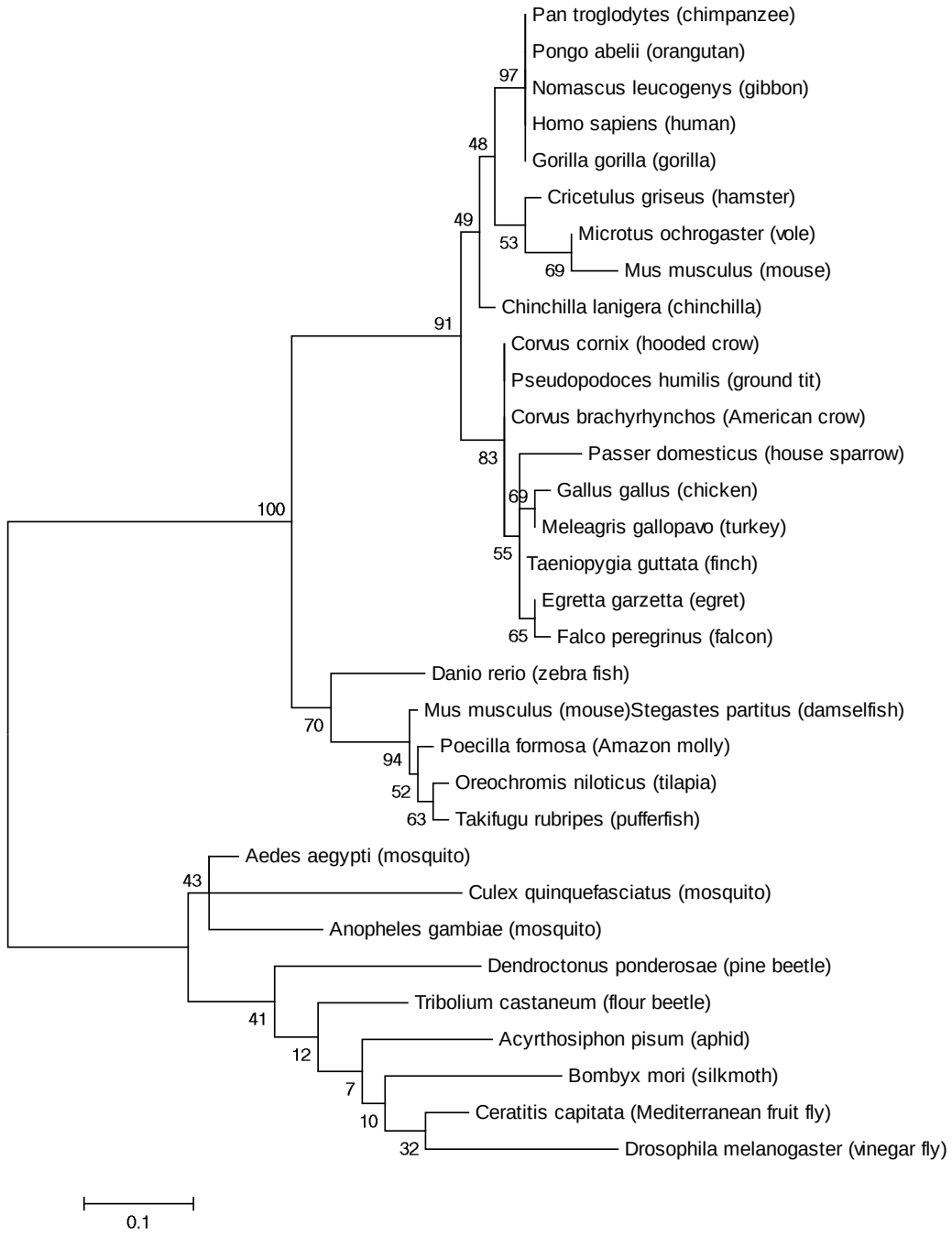
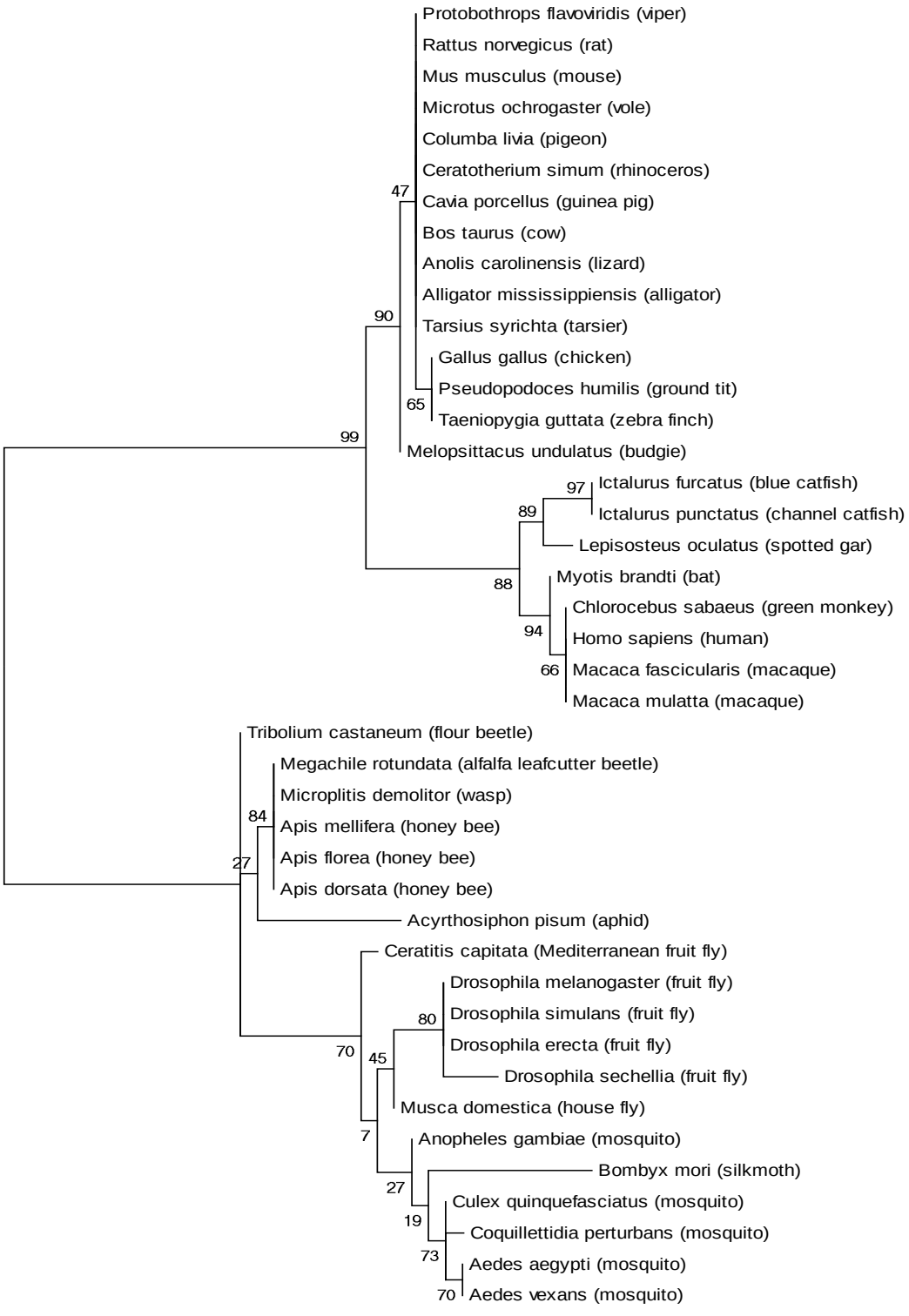
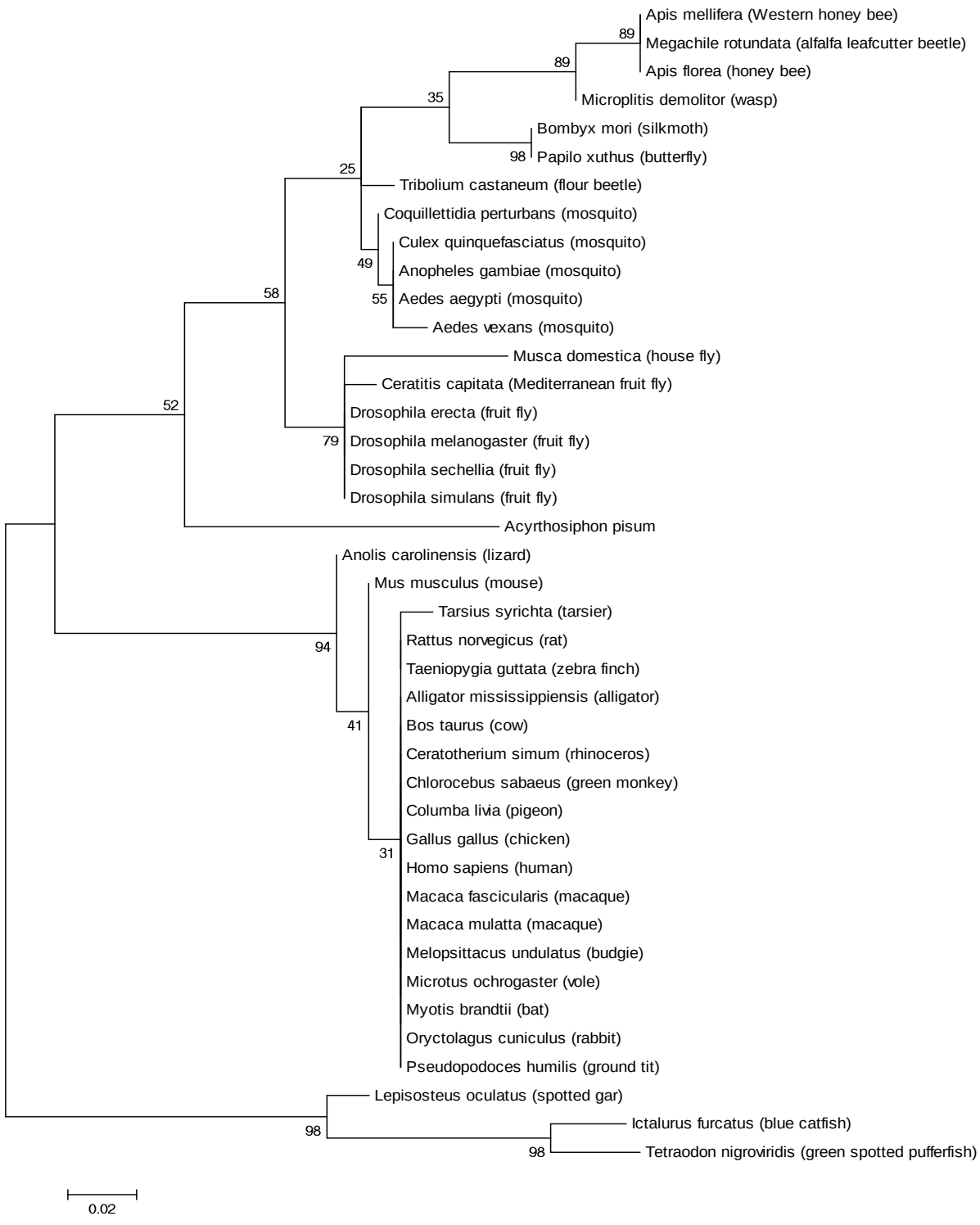


Figure S2. Maximum likelihood neighbour-joining phylogenetic trees of the β3 integrin. Numbers indicate the percent maximum likelihood (the probability that the hypothesized evolutionary history would give rise to the observed data set).

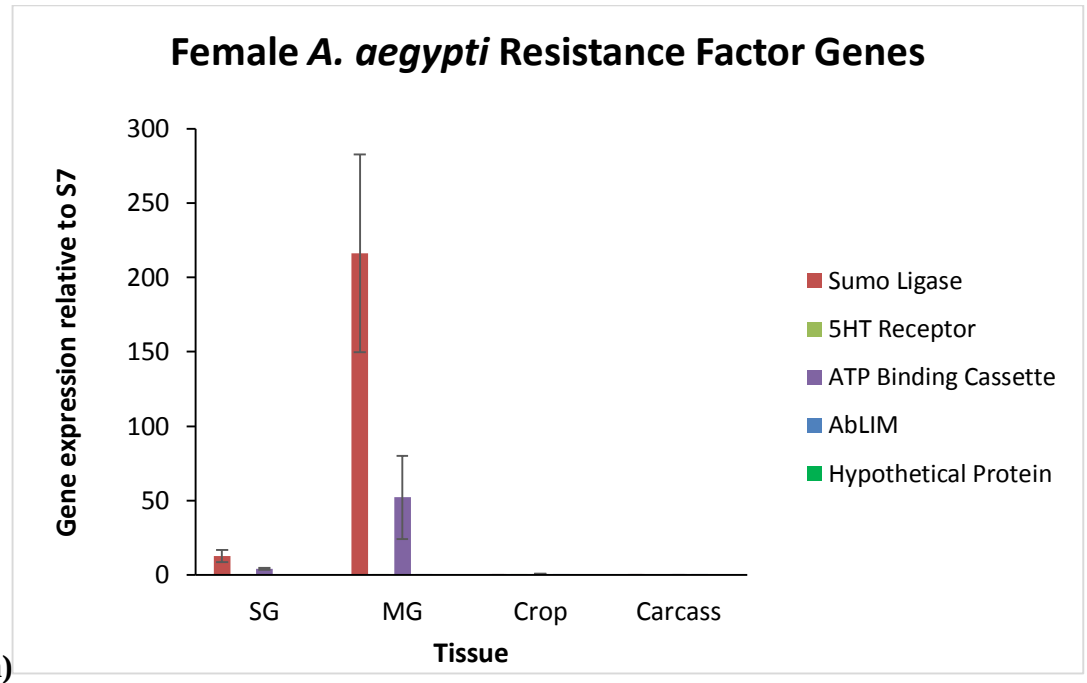

Protobothrops flavoviridis (viper)
Rattus norvegicus (rat)
Mus musculus (mouse)
Microtus ochrogaster (vole)
Columba livia (pigeon)
Ceratotherium simum (rhinoceros)
47
Cavia porcellus (guinea pig)
Bos taurus (cow)
Anolis carolinensis (lizard)
Alligator mississippiensis (alligator)
90
Tarsius syrichta (tarsier)
Gallus gallus (chicken)
Pseudopodoces humilis (ground tit)
65
Taeniopygia guttata (zebra finch)
99
Melopsittacus undulatus (budgie)
97
Ictalurus furcatus (blue catfish)
89
Ictalurus punctatus (channel catfish)
Lepisosteus oculatus (spotted gar)
88
Myotis brandti (bat)
Chlorocebus sabaeus (green monkey)
94
Homo sapiens (human)
66
Macaca fascicularis (macaque)
Macaca mulatta (macaque)
Tribolium castaneum (flour beetle)
Megachile rotundata (alfalfa leafcutter beetle)
Microplitis demolitor (wasp)
84
Apis mellifera (honey bee)
27
Apis florea (honey bee)
Apis dorsata (honey bee)
Acyrthosiphon pisum (aphid)
Ceratitis capitata (Mediterranean fruit fly)
Drosophila melanogaster (fruit fly)
80
Drosophila simulans (fruit fly)
70
Drosophila erecta (fruit fly)
45
Drosophila sechellia (fruit fly)
Musca domestica (house fly)
7
Anopheles gambiae (mosquito)
Bombyx mori (silkmoth)
27
Culex quinquefasciatus (mosquito)
19
Coquillettidia perturbans (mosquito)
73
Aedes aegypti (mosquito)
70
Aedes vexans (mosquito)
0.05

a)

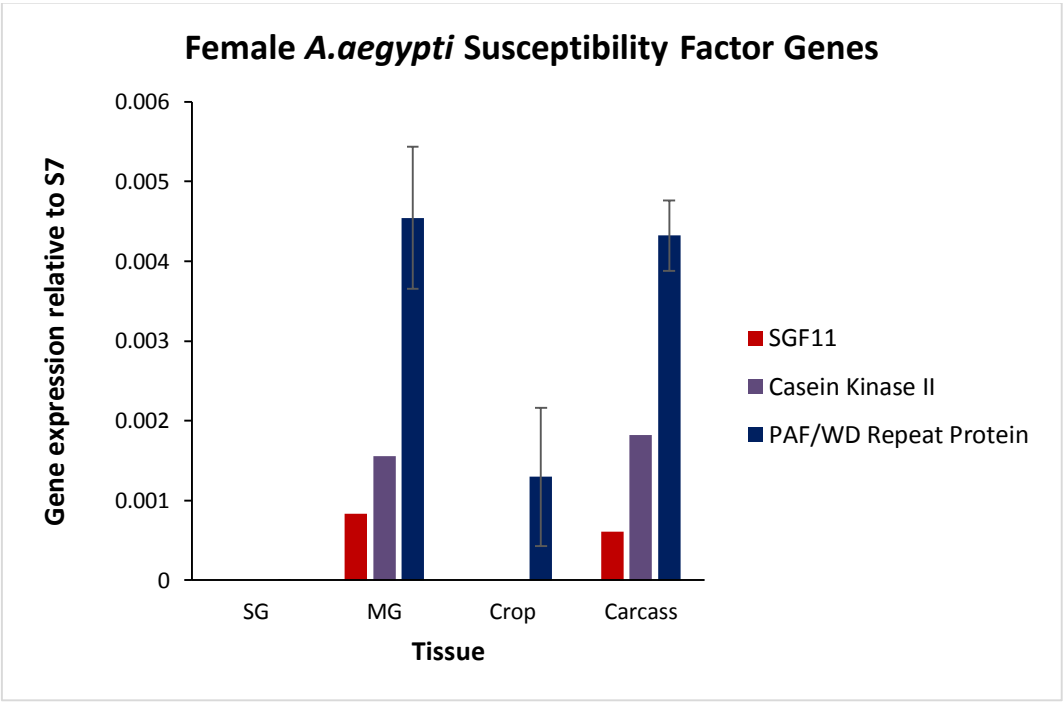


b)

Figure S3. Maximum likelihood neighbour-joining phylogenetic trees of the clathrin coat adaptor proteins a) AP1 and b) AP3. Numbers indicate the percent maximum likelihood (the probability that the hypothesized evolutionary history would give rise to the observed data set).

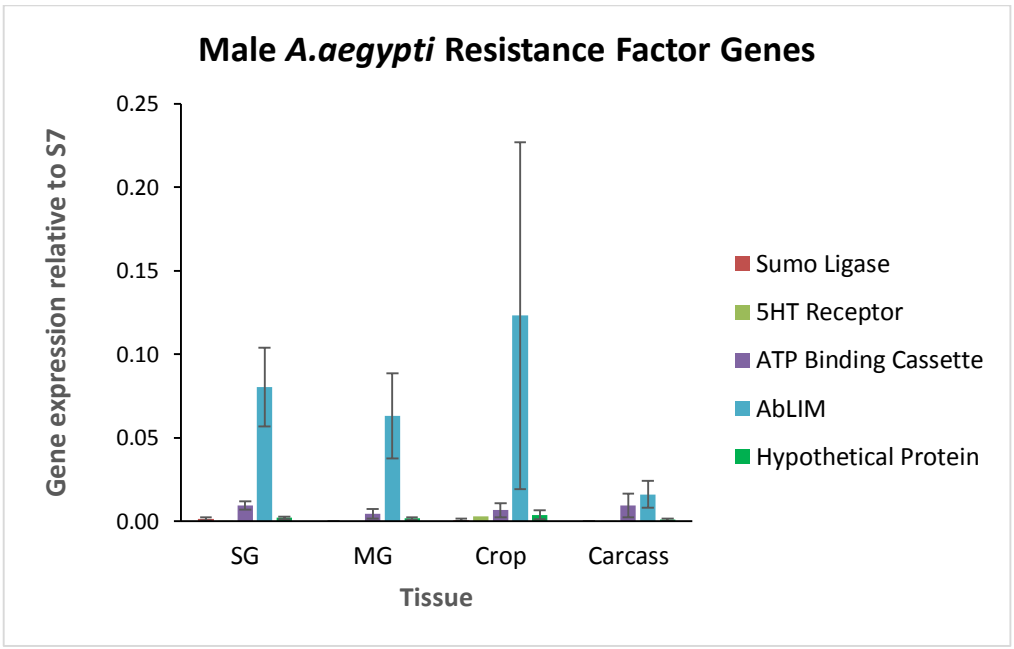

Female A. aegypti Resistance Factor Genes
Gene expression relative to S7
300
250
200
150
100
50
0
SG
MG
Crop
Carcass
Tissue
Sumo Ligase
5HT Receptor
ATP Binding Cassette
AbLIM
Hypothetical Protein

a)


Female A.aegypti Susceptibility Factor Genes
Gene expression relative to S7
0.006
0.005
0.004
0.003
0.002
0.001
0
SG
MG
Crop
Carcass
Tissue
SGF11
Casein Kinase II
PAF/WD Repeat Protein

b)

c)



d)

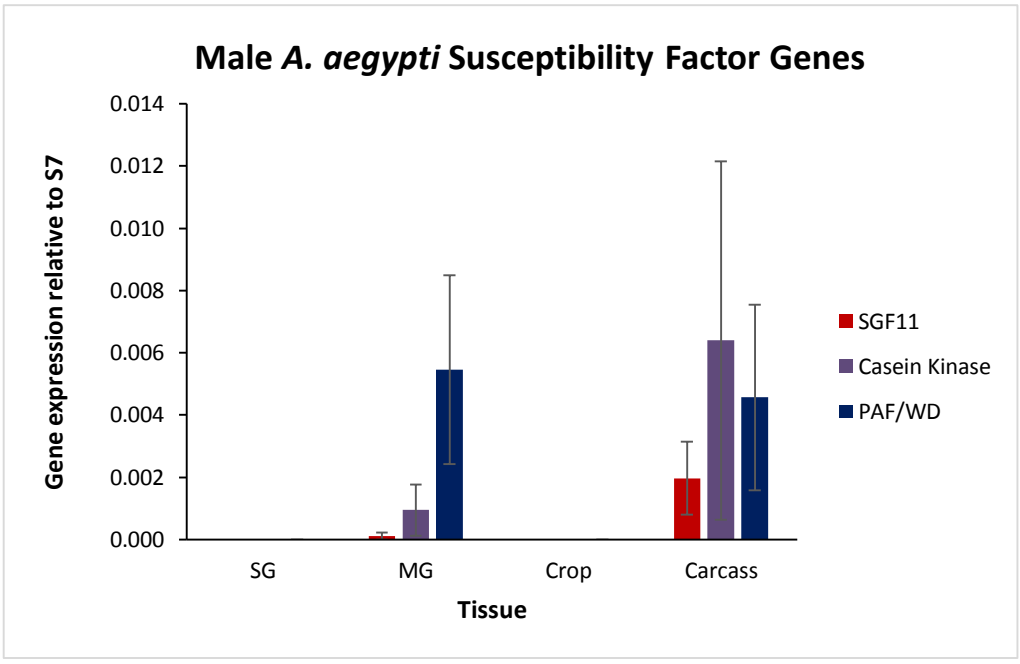


Figure S4. qRT-PCR data of a) resistance factor genes and b) susceptibility genes in *A. aegypti* females and c) resistance factor genes and d) susceptibility factor genes in *A. aegypti* male mosquitoes. Note the scales of the Y axes differ among figures.

Table S1. Percent identities of the clathrin coat adaptor proteins a) AP1 and b) AP3 to *Aedes aegypti*.

a)

Gene	Type of organism	Species	Percent identity to *Aedes aegypti* (nucleotide)	Percent identity to *Aedes aegypti* (protein)
AP1	Mosquitoes	*Culex quinquefasciatus*	75.3	100
		Aedes vexans	93.3	99.0
		Coquillettidia perturbans	85.6	99.1
		Anopheles gambiae	79.5	98.8
	Flies	*Drosophila melanogaster*	73.5	92.0
		Drosophila simulans	78.1	92.0
		Drosophila erecta	77.8	92.0
		Drosophila sechellia	78.1	92.0
		Ceratitis capitata	67.3	90.8
		Musca domestica	72.9	91.3
	Moth	*Bombyx mori*	67.0	92.4
	Butterfly	*Papilio xuthus*	70.0	92.7
	Bees	*Apis mellifera*	66.0	91.0
		Apis florea	66.1	91.0
		Megachile rotundata	66.8	93.7
	Wasp	*Microplitis dermolitor*	70.8	94.6
	Aphid	*Acyrthosiphon pisum*	65.9	88.3
	Beetle	*Tribolium castaneum*	75.8	98.2
	Fish	*Lepisosteus oculatus*	71.4	82.9
		Ictalurus furcatus	66.9	80.2
		Tetraodon nigroviridis	N/A	80.2
	Birds	*Gallus gallus*	65.1	87.4
		Pseudopodoces humilis	64.7	87.4
		Taeniopygia guttata	64.3	87.4
		Melopsittacus undulatus	65.3	87.4
		Columba livia	64.6	87.4
	Reptiles	*Alligator mississippiensis*	64.7	87.4
		Anolis carolinensis	64.7	87.4
	Mammals	*Mus musculus*	63.5	86.5
		Rattus norvegicus	63.9	87.4
		Microtus ochrogaster	64.8	87.4
		Oryctolagus cuniculus	63.3	87.4
		Myotis brandtii	62.7	87.4
		Bos taurus	63.0	87.4
		Ceratotherium simum	71.8	87.4
		Tarsius syrichta	66.2	86.5
		Chlorocebus sabaeus	64.8	87.4
		Macaca fascicularis	64.1	87.4
		Macaca mulatta	65.3	87.4
		Homo sapiens	64.8	87.4

b)

Gene	Type of organism	Species	Percent identity to *Aedes aegypti* (nucleotide)	Percent identity to *Aedes aegypti* (protein)
AP3	Mosquitoes	*Culex quinquefasciatus*	84.2	100
		Aedes vexans	88.6	100
		Coquillettidia perturbans	79.7	94.0
		Anopheles gambiae	74.3	98.4
	Flies	*Drosophila melanogaster*	70.5	94.2
		Drosophila simulans	79.9	94.2
		Drosophila erecta	79.3	94.2
		Drosophila sechellia	79.1	93.2
		Ceratitis capitata	62.8	96.3
		Musca domestica	64.9	96.3
	Moth	*Bombyx mori*	62.1	92.1
	Butterfly	*Papilio xuthus*	59.9	92.1
	Bees	*Apis mellifera*	67.5	92.7
		Apis florea	61.4	92.7
		Apis dorsata	69.4	92.7
		Megachile rotundata	69.7	92.7
	Wasp	*Microplitis dermolitor*	67.5	92.1
	Aphid	*Acyrthosiphon pisum*	67.1	89.0
	Beetle	*Tribolium castaneum*	65.9	92.7
	Fish	*Lepisosteus oculatus*	71.4	79.1
		Ictalurus punctatus	60.8	78.5
		Ictalurus furcatus	64.4	78.0
		Tetraodon nigroviridis	58.8	N/A
	Birds	*Gallus gallus*	58.6	76.4
		Pseudopodoces humilis	60.4	76.4
		Taeniopygia guttata	58.5	76.4
		Melopsittacus undulatus	60.4	75.9
		Columba livia	59.6	73.3
	Reptiles	*Alligator mississippiensis*	60.2	76.4
		Anolis carolinensis	57.2	76.4
		Protobothrops flavoviridis	58.5	76.4
	Mammals	*Mus musculus*	58.2	76.4
		Rattus norvegicus	57.8	76.4
		Microtus ochrogaster	58.4	76.4
		Cavia porcellus	59.9	76.4
		Oryctolagus cuniculus	57.9	76.4
		Myotis brandtii	58.3	76.4
		Bos taurus	57.8	76.4
		Ceratotherium simum	57.9	76.4
		Tarsius syrichta	57.9	75.9
		Chlorocebus sabaeus	59.1	76.4
		Macaca fascicularis	59.0	76.4
		Macaca mulatta	59.8	76.4
		Homo sapiens	58.8	76.4