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Vertical Transmission of Zika Virus in *Monodelphis domestica*

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VERTICAL TRANSMISSION OF ZIKA VIRUS IN *MONODELPHIS DOMESTICA*

A Thesis

by

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ABSTRACT

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Zika Viral infection in pregnant women may lead to infants born with microcephaly and other neurological complications, making Zika viral research imperative. Prior research demonstrated *Monodelphis domestica* to be a unique animal model for Zika virus studies, and viral proteins were detected in brain tissue of juveniles after intracerebral inoculation of infants. We wanted to determine if we can detect a viral protein, non-structural protein 1 (NS1), in brain tissue after intraperitoneal inoculation and we found it to be possible through immunohistochemistry (IHC) analysis. We also investigated if inoculated dams (mothers) would give rise to Zika positive pups (natural inoculation), and we found it to be possible. Further, we compared ELISA assay results to IHC results and found a significant correlation. Our IHC results compared experimentally inoculated dams to naturally infected pups and found that there was no significant difference in the abundance of NS1 in either population. Comparing IHC results of pups of three different ages, we found that pups at 8 weeks had the highest levels of NS1, while NS1 was negligible in 22-week-old and 30-week-old pups.

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CHAPTER I

INTRODUCTION

In 1952, a captive rhesus macaque developed a fever in the Ziika Forest of Uganda, and the infectious agent was named Zika virus. In 1954, there were reports of sporadic cases of Zika virus infections in Africa and Asia. In just two years, the virus had broadened its hosts from non-human primates (NHPs) to human beings. In 2007, the first Zika virus epidemic was reported to affect more than 73% of the population in Yap Island, Micronesia. The same strain of the virus had infected 11% of the population in the French Polynesian Islands via tourists by the end of 2013. In 2014, the virus spread to Chile. The virus was first detected in Brazil in 2015, in the blood samples of patients expressing symptoms like those caused by infection by dengue virus. By 2016, Brazil reported the first laboratory confirmed Zika virus death and 1,326 confirmed cases of microencephaly through congenital infection (Gubler et al., 2017).

Microcephalic newborns are characterized by an abnormally small head. The reduced size of the cephalic perimeter may be due to underdevelopment and even destruction of neural cells. It is the most serious and irreversible neurological complication caused by the virus. In addition to microencephaly, widely open sylvian fissure, reduced size of cerebellum and brain stem, internal hydrocephalus characterized by cerebrospinal fluid buildup and pressure, agyria or the lack of brain folds and grooves in the fetal brain, calcification in cortical and

subcortical white matter is some of the grave anatomical modifications previously reported when the tissue samples of aborted fetuses were analyzed. Zika virus research is imperative for addressing this public health problem, particularly because the demographic of people it affects most severely is newborn children (Gubler et al., 2017).

A study conducted with 216 infants with mothers infected with Zika virus reported that 28.7% scored below average according to the Bayley-III tests and neurodevelopmental tests conducted (Nielsen-Saines et al, 2019). The tests assessed the infants in their cognitive, motor, and lingual functioning. Eight (3.7%) of the 216 infants had microcephaly, two were born with a normal head size but developed microcephaly in the first years of their lives, one was born with microcephaly but later developed a normal head size, and one was born with cranial synostosis, but with surgery developed a normal brain structure (Nielsen-Saines et al., 2019).

Adults may also be impacted by Zika virus infection. Guillain-Barre Syndrome is an autoimmune polyradiculoneuropathy that may be caused by Zika virus infection and is characterized by both pinched nerves and damaged, malfunctioning nerves. It may also lead to respiratory disorders and eventual death. Other disorders in adults include transverse myelitis characterized by a high count of Zika virus RNA in urine, serum, and cerebrospinal fluid; and meningoencephalitis which is inflammation in the meninges of the brain. (Bautista, 2019)

Transmission of Zika happens mainly through mosquitoes of the *Aedes* genus, however cases of horizontal (sexual) and vertical transmission have been observed in humans and experimentally in murine models. (Vue and Tang, 2021; Wrinkler et al., 2017) However, it is deemed unethical to experiment on humans, and murine models have limitations. NHP, another animal model used commonly in Zika virus research, however, they are costly to purchase and maintain. *Monodelphis domestica,* also known as gray short-tailed opossums, are marsupials that

are less expensive than NHPs and do not pose the same limitations as mouse models. They are highly fecund and docile in captivity.

Prior research established that infant *Monodelphis domestica* can be infected with Zika virus via intra-cerebral inoculation (Thomas et al., 2019). That result established gray short-tailed opossums as a potential animal model for Zika virus studies. However, that study was limited in scope. The organ inoculated was the brain, the animals were inoculated shortly after birth, and the tissue tested to prove the persistence of infection was also the brain. In this study, we explore the persistence of Zika virus on brain tissue of *Monodelphis domestica* that were inoculated intra-peritoneally as juveniles. We also compared the extent of brain viremia in terms of the amount of viral protein with levels of viral antibody detected in animals inoculated experimentally and in animals that became infected naturally by vertical transmission from their mothers. The animals that became infected naturally were pups born of mattings between an experimentally inoculated mother and a naive father. We also compared the infection in pups of different ages to determine how antibody levels in the blood serum of the infected pups compare to the actual presence of the viral protein in their tissues as they grow older.

CHAPTER II

LITERATURE REVIEW

Zika virus genome, proteins, and replication

Zika virus is a flavivirus, a single stranded positive sense RNA virus which is mosquito borne. The RNA encodes three structural and seven non-structural proteins making up one polyprotein (Rodrigues de Sousa, 2021). The three structural proteins include the capsid (C), pre-membrane (prM) and envelope (E) which make up the external structure that encases the genetic material. The nonstructural proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. These proteins are crucial for the propagation of the virus in host cells. The Zika virus has been detected in human placental, neuronal, skin, uterine, and testicular tissue; amniotic fluid; and peripheral blood mononuclear cells (PBMCs) (Estévez-Herrera, 2021).

The virus invades host cells via receptor mediated endocytosis. The envelope protein then goes through a confirmational change causing the viral membrane and host membrane to fuse, and the genetic material encased in the capsid is then released into the cytoplasm of the host cells. Viral RNA then disassociates from the capsid (Wang et al., 2018).

Replication of the virus occurs in replication compartments derived from the endoplasmic reticulum (ER), constituted of vesicle packets and complex membrane structure. NS1, the first non-structural protein to be discovered, associates with lipids and forms a homodimer in

cells that are vital for initial viral replication and later stages of infection. The NS1 protein remodels the ER where the replication of the virus takes place and where mRNA is translated into a single polyprotein that forms vesicle packets. The remodeling forces the ER to create invaginations that form the vesicle packets; this phenomenon does not naturally occur in the ER and is induced by NS1 binding to the ER (Wang et al., 2018; Ci et al., 2019; Song et al., 2016).

The other non-structural proteins (NS2A-NS5) form a replication complex and attach to the cytoplasmic side of the ER. The vesicles are cleaved by viral proteases (NS5 and NS3) and host cell proteases to form the structural and non-structural proteins and are transferred to the Golgi apparatus for glycosylation and consequent maturation. Mature viruses and subviral particles, without the genetic material, and capsid are released from the host cells by exocytosis, allowing the propagation of the virus in the body of the host (Wang et al., 2018; Ci et al., 2019).

Additionally, NS2A, NS2B, NS4A and NS4B are scaffolding proteins for the replication complex, whereas NS1 and NS4B and structural protein E (envelope protein) inhibit type I interferon production and cause immune evasion. NS1 is a celebrated antigenic marker for viral detection (Song et al., 2016; Vivir and Malissen, 2005). In this study we will employ ELISA to detect antibodies to Zika virus in blood serum, and immunohistochemistry to detect NS1 in tissues (Wang et al., 2018; Thomas et al., 2019)

Zika virus transmission studies

The primary mode of transmission of Zika virus is through the mosquitos of the *Aedes* genus (Yu et al., 2021). The virus is spread when a mosquito bites an infected individual and then an uninfected individual, transmitting infected blood from one to the other. Zika virus also

can be transmitted from person to person horizontally or vertically. Horizontal transmission occurs sexually, and vertical transmission occurs via passage of the virus from mother to progeny in utero. (Lanicotti et al., 2007; Monel et al., 2017).

Sexual transmission of Zika virus was reported in the United States in 2017 (Vue and Tang, 2021). Of the 46 cases reported, the majority were symptomatic male to female transmission. However, cases of symptomatic female to male and asymptomatic male to female transmission were also reported. (Vue and Tang, 2021) However, transmission studies on murine models with a knocked-out interferon gene revealed only infected male to female sexual transmission (Wrinkler et al., 2017).

Vertical transmission of Zika virus has been observed in humans and in murine models. Fathers do not directly transfer Zika to their children via the sperm but can infect the mother sexually and the resultant child (Vue and Tang, 2021; Wrinkler et al., 2017).

Murine models provide useful information about viral transmission and pathogenesis. However, wild-type immunocompetent mice do not support Zika virus infection, so most mice used in Zika viral research are transgenic immune-deficient and may not be a fully valid representation of viral processes in humans. Another model used for viral research is NHPs (non-human primates), which have biological systems that most closely resemble human systems, including those that involve responses to pathogens. However, they are costly to maintain, and a much longer time span is required to investigate vertical transmission because they have long gestation periods.

Monodelphis domestica as animal models

This study uses *Monodelphis domestica* as a model organism, a non-traditional model for Zika viral infection, which can overcome the limitations of murine and NHP models. These animals, commonly known as gray short-tailed opossums or laboratory opossums, are native to Brazil and some surrounding countries. They are approximately three times the size of mice, making it practical to collect considerably larger volumes of blood at a given time, and from repeated samplings for longitudinal assessments. They reproduce abundantly, and at birth they are developmentally like 6-week human embryos, making them a unique laboratory animal for developmental research (VandeBerg and Williams-Blangero, 2010).

Zika immune invasion and *M. domestica* and immune response

Interferons are proteins that are released in the body in response to a viral infection. Interferon-α (IFN-α) gene is associated with suppression of immune evasion in mice. Mice have 14 IFN-α genes, humans have 13 and *Monodelphis* have seven (Wong et al., 2006; van Pesch et al., 2004; Belov et al., 2007). Mice are naturally able to suppress immune evasion of Zika virus, unlike humans and *Monodelphis.,* It is unclear how they do so, but perhaps their additional IFN-α gene is involved. IFN- α falls under a family of Type 1 genes. Type 1 protein (a cytokine or intracellular messenger immunoregulation proteins) in response to STAT2 which is an adaptor protein suppresses immune evasion in mice, however, in humans Zika virus can propagate as Zika is able to suppress STAT2. (Rodrigues de Sousa, 2021)

Mice are born with an adaptive immune system, but they do not have an innate immune system at birth. An adaptive immune system holds “memory” of germs that have been encountered. It is initially slower than the innate immune system to respond, but it adapts and

changes to provide better support in case of a similar viral attack. Some mice at birth are immune deviant which means that they can have immune systems that function like that of an adult, helping them fight off viral attacks. (Vivir and Malissen, 2005; Adkins et al., 2004)

Newborn opossums borrow antibodies (passive immunity) from their mothers and do not have their own immune system; they develop one as they grow older (Samples and Vandeberg, 1986). The innate immune system works faster, is non- specific and activates proteins and natural killer cells to eliminate pathogen. However, it is not capable of preventing Zika viral infection in *Monodelphis* and humans (Vivir and Malissen, 2005).

Previous research of Zika viral infection in *M. domestica*

Prior studies with *Monodelphis domestica* have demonstrated their ability to be persistently infected with Zika virus when inoculated intra cerebrally shortly after birth, providing researchers an opportunity to study the pathogenesis of the virus in a new model (Thomas et al., 2019). Results from prior research also have established that juvenile *Monodelphis domestica* can be persistently infected via intraperitoneal inoculation of Zika virus and that the virus can be transmitted vertically from inoculated dams to their progeny (J.L. VandeBerg, personal communication). However, vertical transmission has not been verified by a rigorous prospective experimental study. This thesis will determine in a prospective study if Zika viral protein NS1 is detectable in brain tissues of pups born to mothers that had been experimentally infected with Zika virus.

Zika virus and the brain

Vertical transmission of Zika virus can lead to several brain malformations like microencephaly, in infants. Previous research suggests that Zika viral infection in infants impairs

neurogenesis (cell growth) and causes cell death and primarily targets neural progenitor cells (NPCs). NPCs are stem cells that may become specialized in due time. In the first trimester of pregnancy, the brain of the unborn infant is primarily composed of NPCs. The P13K-Akt_mTOR pathway, which is essential for NPC differentiation, is suppressed by Zika virus proteins NS4A and NAS4B, the replication of which leads to impairment of brain tissue development. (Russo and Beltrao-Braga, 2017).

In this study we limited the region of interest to the cerebellum and the cerebral cortex in sagittal sections of *Monodelphis domestica*. The sagittal section was chosen as it provides access to both the cerebellum and the cerebral cortex at the same time. We are interested in viral infection in the cerebellum because previous research demonstrated the presence of Zika virus in the cerebellum via IHC and in-situ hybridization methodologies. Both replication proteins, NS1 via IHC and NS5 via in-situ hybridization, were detected in the cerebellum, suggesting the utility of this tissue for detecting Zika virus infection (Thomas et al., 2019).

We also assessed infection of the cerebral cortex, because research done in immuno-competent mice revealed that Zika viral infection greatly impacts the cerebral cortex. That conclusion was reached by detection of the Zika virus envelope protein in the cerebral cortex. The sizes and weights of the infected neonatal mice were also decreased, giving us reason to screen this area of *Monodelphis* brain tissue for the presence of virus (Li et al., 2018).

Statement of problem

Infection of Zika virus leads to congenital neurological disease like microencephaly which has led to developmental pathologies in infants. It also may cause Guillain-Barre syndrome in adults in rare cases. The most severe consequence of Zika viral infection affects the

infant population and therefore, thorough investigation of its pathology is imperative. Previous research has shown *Monodelphis domestica* to be an immune-competent animal model for Zika virus research, however, rigorous transmission studies have not been conducted with this species. It is not well researched if Monodelphis can transmit the virus and naturally infect their mates or progeny. Levels of infection have been assessed by IHC and ELISA, however, no systematic correlation studies had been done to assess levels of Zika antibodies in blood samples to levels of viral proteins detected in the tissues of the animals. Furthermore, levels of viral proteins in experimentally inoculated animals have not been compared to levels in naturally infected animals. These comparisons will help us understand the extent of the transmission of the virus and its persistence *Monodelphis domestica.* The understanding of how natural infection of *Monodelphis* infants affects antibody levels is also not well understood.

Purpose of this study

This study aims to assess the correlation between IHC and ELISA results in detecting Zika virus infection. We do not expect to see a perfect correlation but a general similarity in the trend of the two data sets. We also attempt to understand the extent of viral infection in the brain tissue of experimentally infected dams and naturally infected pups of the inoculated dams. We expect to see stronger signals in the experimentally inoculated dams than in naturally infected pups. We are interested in determining if IHC results support the conclusion from ELISA results that experimentally infected dams can produce litters in which some but not all pups are infected. It is important, too, to explore the viral infection through IHC results across different stages of development in naturally infected pups. Based on the observation that ELISA titers decreased the age of the pups increased, we expected the IHC results of pups at 8 weeks to indicate a higher

concentration of viral proteins in the brain tissue of 8-week-old pups than the brain tissue of their older siblings (22 and 30 weeks).

CHAPTER III

MATERIALS AND METHODS

Overall experimental design on which this thesis is based

The IHC conducted for this thesis used a subset of samples collected from animals involved in a major study that will involve three groups of opossums. All animals used in this study were from the LL2 (Large litters 2) stock, which is randomly bred stock. Group 1 animals were assigned to the cage mate experiment. The objective of this group was to determine if Zika virus is airborne or can be transmitted through cage-mate contact, or urine or feces. An inoculated weanling was placed in the same cage as uninoculated littermates for 13 weeks. The inoculations were done intraperitoneally. Four littermates were housed per cage, in a total of 10 cages, with two nest boxes or mason jars in which animals could hide from one another. The four littermates were distinguished by signature ear notches of 3-5 mm in diameter. The right ear of the inoculated animal was notched while the uninoculated animals were identified by notches in the left ear (top, middle and bottom location). Blood was collected by cardiac puncture from all animals before inoculations and at weeks 1, 3, 5, 9, and 13 after inoculations.

Group 2 animals were assigned to the female to male sexual transmission experiment. Ten inoculated females were paired for 2 weeks with four naive males in succession, making a

pool of 40 potentially sexually infected males. *Monodelphis domestica* are induced ovulators, and copulation usually occurs 6-8 days after a female is paired with a male. Blood was collected from the males before pairing and on the day of separation from the female, 14 days after pairing. If the animal was found to be ELISA negative for antibodies, another blood sample was collected on Day 28. If the animal was still negative, another sample was collected on Day 42. Group 3 animals were assigned to the male to female sexual transmission experiment. Ten inoculated males were each paired sequentially for 2 weeks with four females, making a pool of 40 potentially sexually infected females. Blood was collected from females before pairing on Day 0 and, if a litter was not produced, again on the Day 28 (14 days after separation of the pair mates). If a litter was produced, blood was collected from the mother after the litter had been weaned at 8 weeks of age.

For both Group 2 and Group 3, when the pups from each litter reached the age of 13 weeks, blood samples were collected from them. Blood samples were then processed to isolate serum, which was stored at -80 C, and subjected later to ELISA, which was developed to detect antibodies against Zika virus in serum of this species. Most pups were euthanized at that time for collection of tissues for IHC, but some pups were allowed to live longer for repeated blood sampling for ELISAs and tissue collection later.

ELISA

The type of ELISA employed is 'indirect ELISA,' in which microtiter plates are coated with twice clarified inactivated Zika virus extracted from Vero E6 cells, a cell culture lineage developed from green monkey liver cells. The whole virus serves as a set of capture antigens (potentially all the viral proteins) to which all serum antibodies directed against Zika virus can

potentially bind. All other binding sites on the surface of the plates are blocked with a blocking solution (bovine serum albumin), and finally the bound antibodies are detected with a secondary antibody tagged with TMB, a chromogenic derivative of horseradish peroxidase.

The detection of antibodies against whole virus in serum samples i.e., a positive ELISA result, must be followed up by a direct method of virus detection in tissue cells in order to discriminate among the following alternatives: 1) the animal is infected with Zika virus, 2) the animal was previously infected, but cleared the virus, or 3) the ELISA provided a false positive result, and the animal was never infected with Zika virus.

My role in the overall experiment was to help with blood and tissue collections, to participate in periodic group discussions and evaluations of the data as results were obtained, and to become familiar with the ELISA. However, the work described above was conducted primarily by staff in Dr. VandeBerg's laboratory.

The remainder of the Methods and Materials section pertains to the research that I conducted for this thesis.

Animals used in this study

The animals selected for IHC for this thesis are from Group 2, female to male sexual transmission, in which the female opossums were inoculated with virus. A total of fourteen litters were born in this group. I focused on litters 13 and 14, because they had pups that were ELISA positive, negative, and indeterminate (ELISA titer values of >1.000, <0.900, and 0.900 - 1.000, respectively), and the pups were necropsied at different ages. For positive controls, tissues were collected from animals from a separate study where the opossums were experimentally inoculated multiple times. For negative controls, tissues were taken from ELISA negative

breeders whose parents and grandparents also had tested ELISA negative. I studied a total of seven dams and 19 pups, for a total of 26 animals. (**Tables 1-3**)

Table 1. ID numbers and characteristics of dams selected for IHC.

Artificially inoculated Dams	ELISA status at necropsy	ELISA titer at necropsy	Age at necropsy (weeks)
P7893	Positive	24.69	56
P7894	Positive	3.15	56
P7936	Positive	19.35	54
P7937	Positive	52.24	58
P7938	Positive	1.41	54
P7976	Positive	50.22	54
P7978	Positive	2.71	58

Table 2. ID numbers and characteristics of Litter 13, selected for IHC. Note that all pups were ELISA-positive (i.e., titers > 1.00) at 8 weeks of age.

Naturally infected pups (litter #13)	ELISA titer at 8 weeks	ELISA titer at necropsy.	Age at necropsy (weeks)
Q0431	38.14	38.14	8
Q0432	10.87	0.46	30
Q0434	8.92	0.22	22
Q0435	14.16	0.23	30
Q0436	39.14	39.14	8
Q0437	31.14	31.14	8
Q0438	12.42	0.15	30
Q0439	11.1	0.49	30
Q0440	9.89	0.11	30
Q0441	12.7	0.03	30
Q0442	14.09	0.13	22
Q0443	7.55	0.17	30

Table 3. ID numbers and characteristics of Litter 14, selected for IHC.

Naturally inoculated pups' litter (#14)	ELISA titer at 8 weeks	ELISA titer at necropsy.	Age at necropsy (weeks)
Q0444	0.96	0.96	8
Q0446	0.38	0.38	30
Q0448	0.31	0.16	30
Q0450	1.17	1.39	8
Q0451	1.29	1.53	8
Q0452	0.33	0.21	30
Q0453	0.32	0.24	30

Immunohistochemistry

The research conducted for this thesis involved IHC of brain samples obtained from some of the inoculated females that produced litters and from some of the progeny, and analysis of the data to address the questions defined under Purpose of this Study.

The antigen selected as the target for detection by IHC is non-structural antibody (NS1), which is essential for viral replication in infected cells, and which was previously identified as the preferred viral protein for detecting Zika virus in *Monodelphis domestica* tissues by IHC. In addition, prior research revealed the presence of NS1 in brain, spleen, liver, kidney, and reproductive organs of experimentally infected *Monodelphis domestica.*

Brain tissues were selected because 1) Zika virus is known to be neurotropic in humans and in mice and to cause brain pathologies, and 2) an abundance of Zika virus NS1 was observed in brain of experimentally infected *Monodelphis domestica* in previous research. The regions of focus were the cerebellum and cerebral cortex.

IHC is a procedure that involves staining tissues to scan for and image tissues utilizing wide field florescence microscopy. The tissue is frozen with a freezing compound and sectioned using a cryostat. Thin sections of tissue make viral detection easier and image quality higher. Therefore, this study used sections of 10 um in thickness. The sections were mounted on slides which were washed with a mixture of PBS (phosphate buffered saline) and BSA (bovine serum albumin) to limit nonspecific binding by the primary antibody. The primary antibody, anti-Zika NS1, was applied, and the slides were incubated overnight. The tissues were also treated with a cellular stain mixture of DAPI (stains the nucleus), and Alexa fluor 488 phalloidin stain (stains the microtubules, which are a part of the cytoskeleton of the cells). Wash Buffer (PBS-TB) constituted of Sterile 1x PBS from the company Gibco with catalogue number: 10010031; 0.1% Tween 20 from Sigma-Aldrich with catalogue number: P9416; 2% Bovine Albumin Fraction V from MP Biomedical catalogue number: 810033; Stored at 4ºC. The Anti-NS1 Primary Antibody was from the company Arigo Biolaboratories with catalogue number: ARG65781; Stored at -20ºC protected from light. Alexa Fluor 546 conjugated secondary antibody from Invitrogen with catalogue number: A11030; Stored at 4ºC protected from light. Alexa Fluor 488 conjugated Phalloidin from Invitrogen with catalogue number: A12379; Stored at -20ºC; protected from light. DAPI from Sigma-Aldrich catalogue number: D9542; Stored at -20ºC; Protected from light.

The slides were then viewed under a florescence microscope under three filters: red, blue, and green. The red filter picks up on the NS1 antibody bound to the NS1 protein and enables visualization of the same. The blue filter enables visualization of the nuclei (DAPI), and the green filter enables visualization of microtubules (Phalloidin stain). A composite image is formed based on the images taken through the three filters to determine the location of NS1

relative to the nuclei and cytoskeleton. The protocol for immunohistochemistry was provided by Dr. Mathew D. Terry. (Quintanilla and Thomas, 2020)

Analysis of IHC images

Four sagittal tissue sections of 10 μm were made from each brain. Three were treated with the primary antibody, and one without it. A total of 14 images were taken per animal. Four images were taken per section treated with primary antibody, two from the cerebral cortex area and two from the cerebellar area. Two images were taken from the section treated without antibody. A total of 364 images were analyzed.

The images were then scored from 0-5 (see Figure 1 for examples of NS1 scores). 0 equates to no detection, 1 is low, 2 is low moderate (between low and moderate), 3 is moderate, 4 is moderate high (between moderate and high) and 5 is high. Depending on the score of each image an average score was calculated from the NS1 observed in the cerebral cortex and an average score was calculated for the cerebellum. These data were used to conduct statistical analyses. The final score used was an average of the score of the cerebral cortex and cerebellum from each animal.

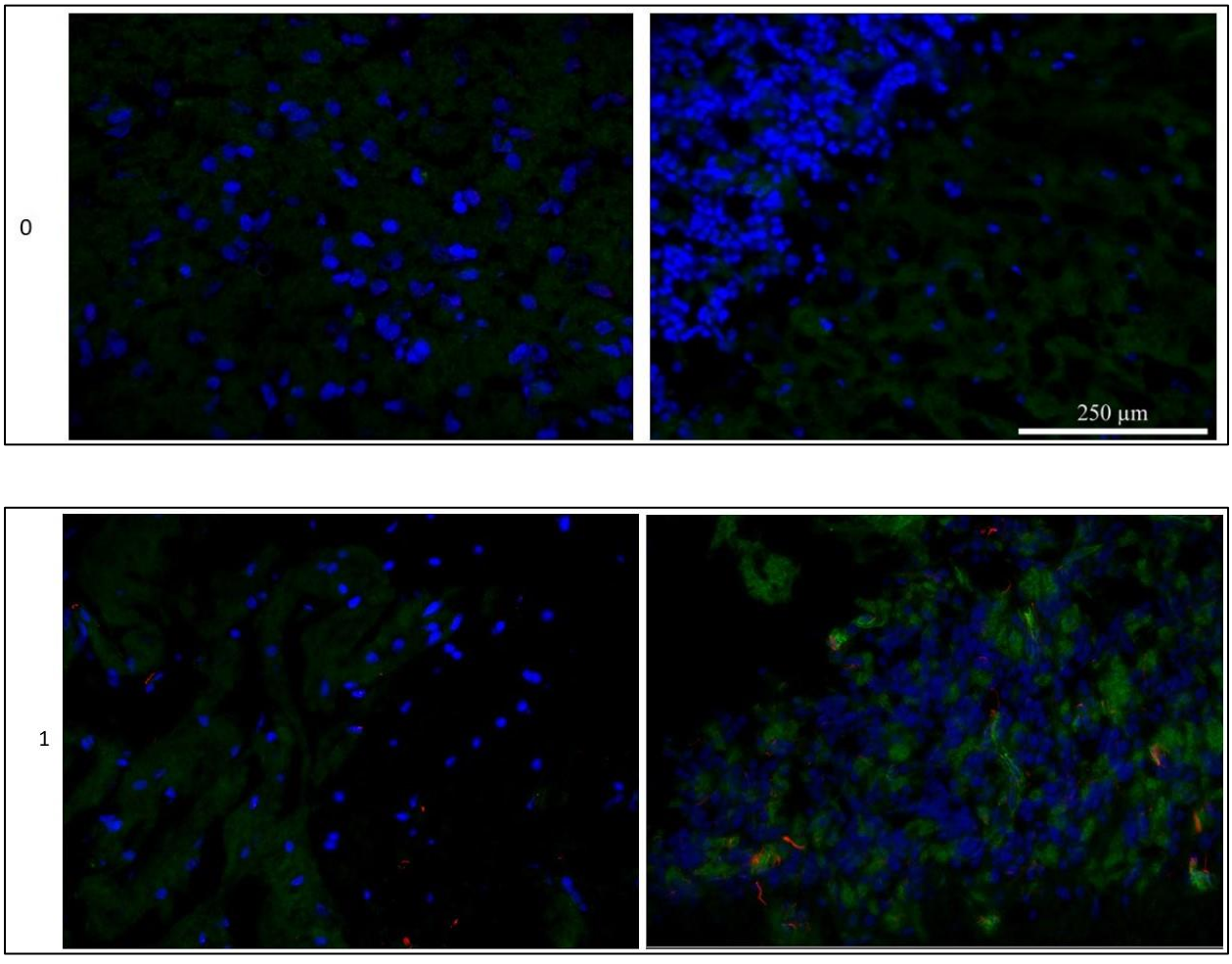
A Welsh's T-test was conducted comparing the IHC results of the seven dams and the 19 pups. This statistical test calculates the average of two populations that have different sample sizes. The ELISA results revealed that the viral antibody levels in the pups decreased with age. We expected to see the same trend in IHC results. A one-way ANOVA test was conducted using the IHC results of the pups based on their age. This test is like the T-test; however, it can compare the means of more than two categories of samples.

CHAPTER IV

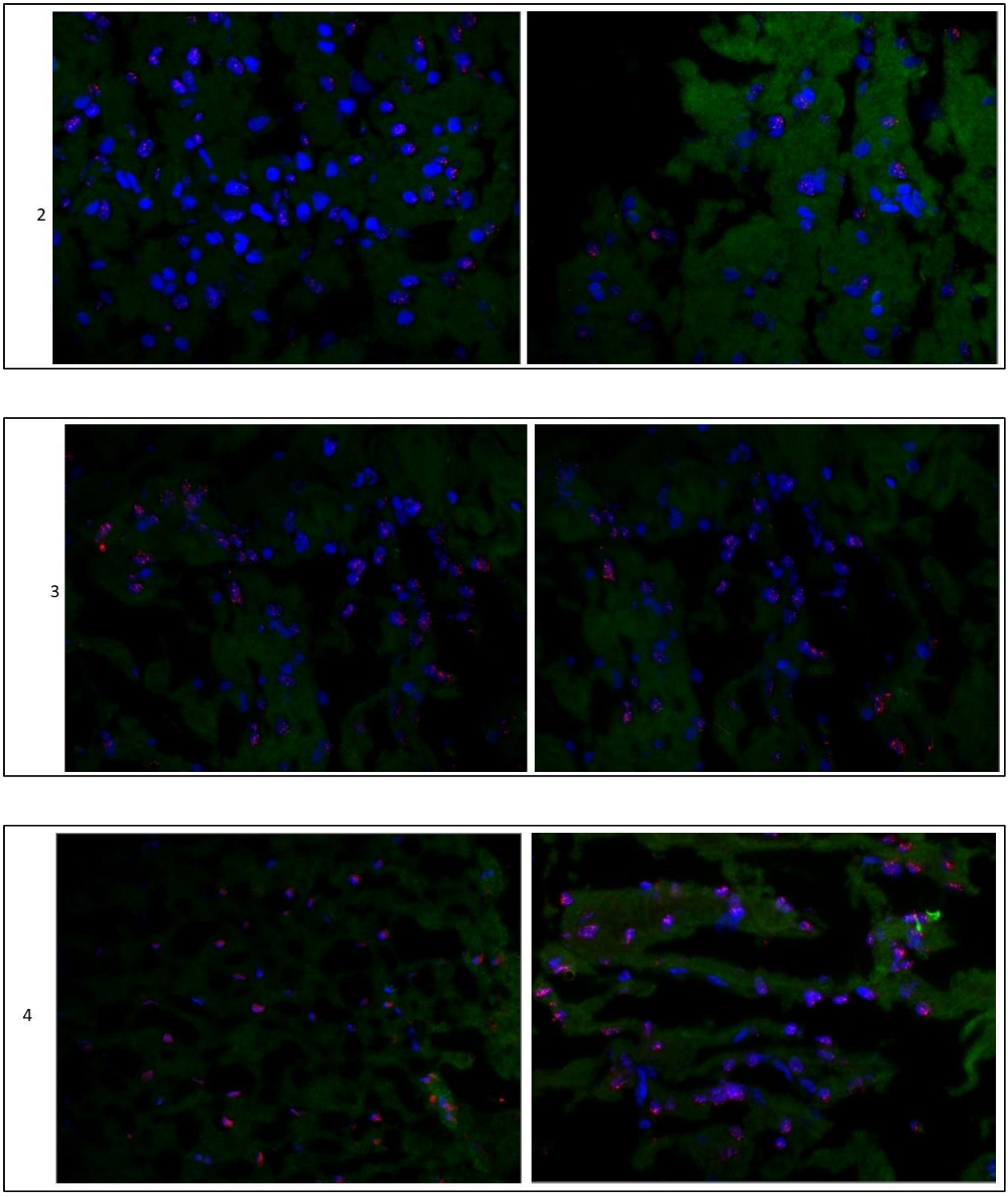
RESULTS

Immunohistochemical analysis

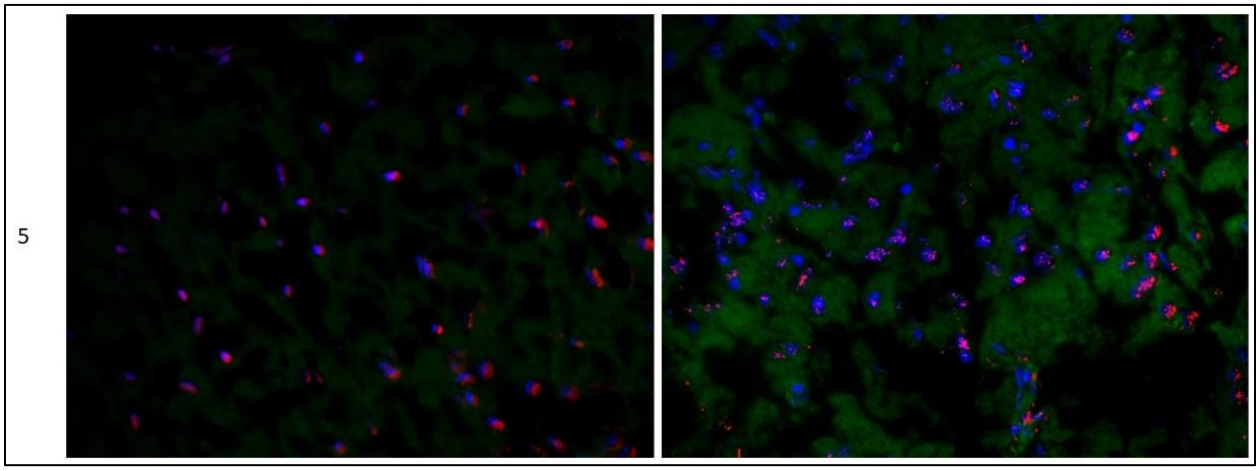
Figures 1.1-1.6 are pictorial examples of the NS1 average score scale 0-5.



Figures 1.1-1.6: Examples of NS1 score. 40X magnification, 250 µm scale bar. The red indicates NS1, while the blue indicates nuclei (DAPI) and the green indicates F-actin (Alexa fluor 488).



Figures 1.1-1.6: Examples of NS1 score. 40X magnification, 250 µm scale bar. The red indicates NS1, while the blue indicates nuclei (DAPI) and the green indicates F-actin (Alexa fluor 488)



Figures 1.1-1.6: Examples of NS1 score. 40X magnification, 250 µm scale bar. The red indicates NS1, while the blue indicates nuclei (DAPI) and the green indicates F-actin (Alexa fluor 488).

One aim was to determine if ELISA titers of Zika viral antibody levels in the serum of *Monodelphis domestica* are correlated with the semi-quantitative NS1 scores that we determined from IHC analysis. The coefficient of correlation was 0.72 when comparing the results of all the 26 animals in the study indicating a strong correlation (**Figure 2.1**), however the coefficient of correlation was 0.62 when comparing the results of JUST the infected animals (**Figure 2.2**). Animals that are ELISA positive have average NS1 scores in the range of 2.25-4.83 (**Tables 4 and 5**), and animals that are ELISA negative have average NS1 scores in the range of 0.00-1.4 (excluding Q0444, with an indeterminate ELISA titer) (**Tables 4 and 5**). The score for Q0444 was 3.12.

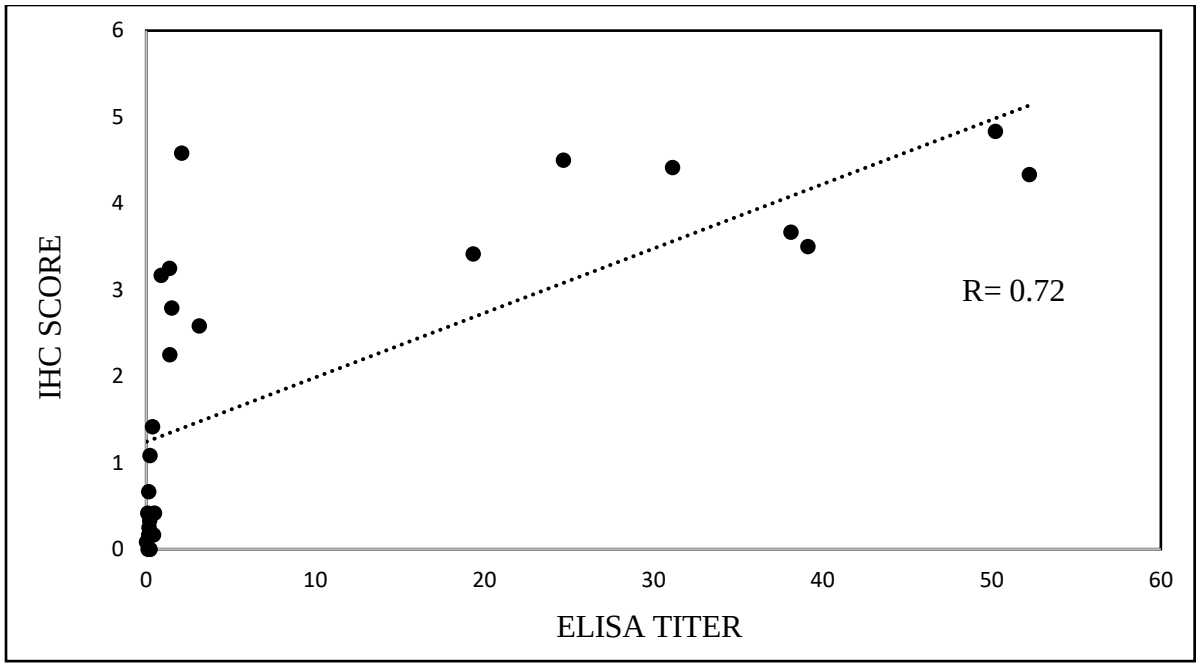


Figure 2.1: Correlation between ELISA titers and IHC scores. R is the coefficient of correlation which is 0.72 for ALL animals.

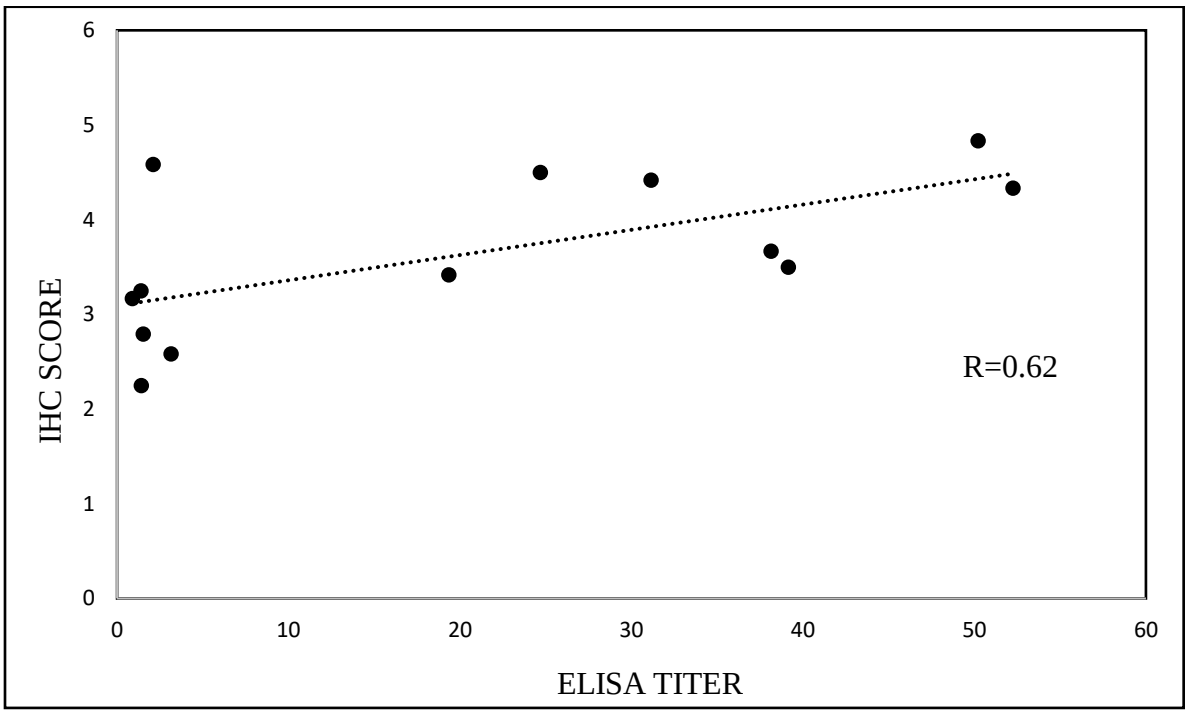


Figure 2.2: Correlation between ELISA titers and IHC scores. R is the coefficient of correlation which is 0.62 when comparing the results of ONLY the infected animals.

Table 4. IHC and ELISA results for dams. The shaded boxes contain the data from animals that had a positive ELISA titer (>1.00=green) or indeterminate ELISA titer (0.90-1.00=yellow) at necropsy.

Experimentally inoculated dams	NS1 average score	ELISA titer at necropsy	Age at necropsy (in weeks)
P7893	4.50	24.69	56
P7894	2.58	3.15	56
P7936	3.42	19.35	54
P7937	4.33	52.24	58
P7938	2.25	1.41	54
P7976	4.83	50.22	54
P7978	4.58	2.71	58

Table 5. IHC and ELISA results for Litters 13 and 14.

Naturally infected pups (litter #13)	NS1 average score	ELISA titer at necropsy	Age at necropsy (in weeks)
Q0431	3.67	38.14	8
Q0432	0.00	0.46	30
Q0434	0.17	0.22	22
Q0435	0.17	0.23	30
Q0436	3.50	39.14	8
Q0437	4.42	31.14	8
Q0438	0.00	0.15	30
Q0439	0.42	0.49	30
Q0440	0.42	0.11	30
Q0441	0.08	0.03	30
Q0442	0.00	0.13	22
Q0443	0.25	0.17	30
Naturally infected pups' litter (#14)	NS1 average score	ELISA titer at necropsy	Age at necropsy (in weeks)
Q0444	3.17	0.96	8
Q0446	1.42	0.38	30
Q0448	0.67	0.16	30
Q0450	3.25	1.39	8
Q0451	2.79	1.53	8
Q0452	0.33	0.21	30
Q0453	1.083	0.24	30

We compared experimentally inoculated dams to 8-week-old pups that to which the virus had been transmitted from their mother. To do this, we conducted Welsh's t-test, which does not assume equal sample size or equal variance. The test resulted in a t-value of 0.706710 which is used to calculate the p-value, which was 0.497634, indicating no significant difference between dams and 8-week-old pups. Dams had an average NS1 score of 3.79 while the 8-week-old pups had an average score of 3.47, the two scores were not significantly different (**Figure 3**). The standard deviation for dams was 1.04 and for 8-week-old pups it was 0.55, suggesting higher variance in the sample of dams. **Figures 4.1 and 4.2** depict the NS1 signal of pup Q0437 by comparison to dams P7893 and P7937 (the average scores of these animals were 4.42 vs 4.50 and 4.33, respectively).

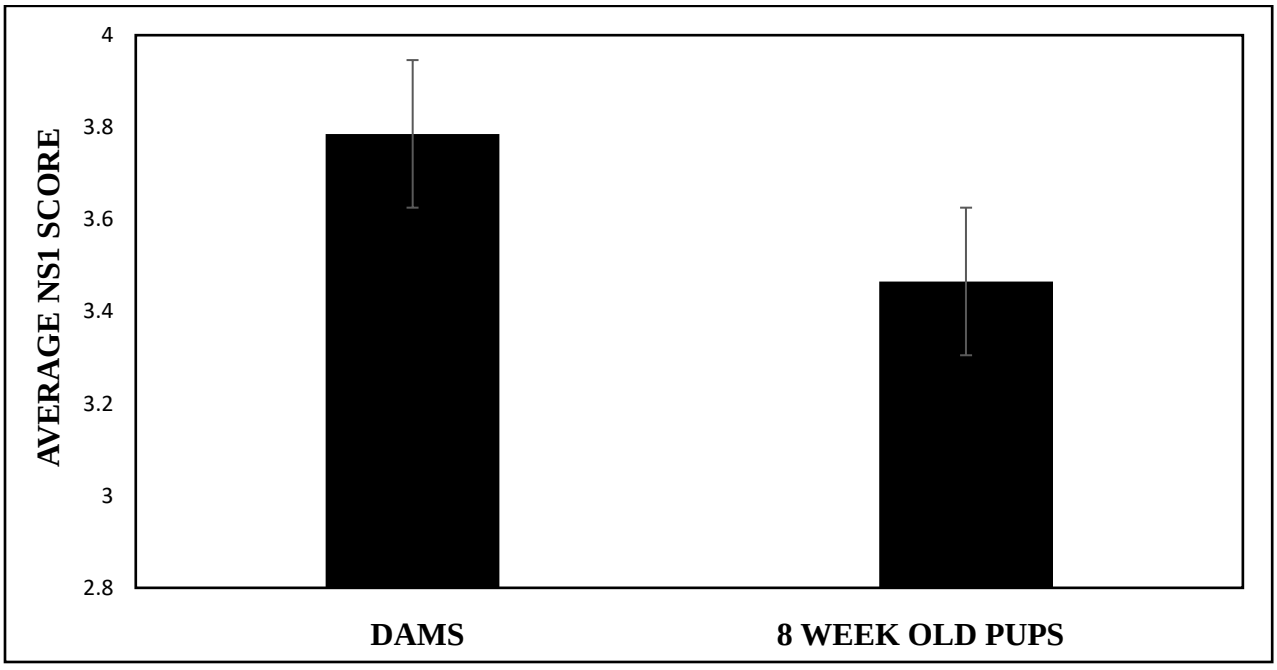


Figure 3: **Comparison of NS1 scores of dams and 8-week-old pups.** No statistical significance was observed between the two samples. The bars represent standard error.

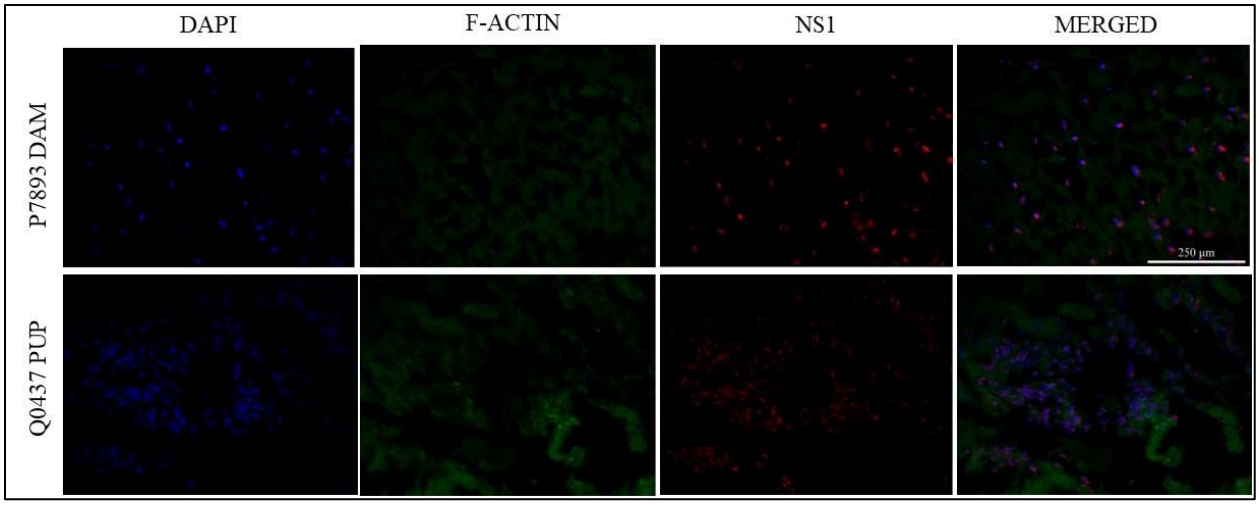


Figure 4.1: Cerebellum of P7893 dam and Q0437 pup. The NS1 score for this image of the dam (top) is 4 and the score for this image of the pup (bottom) is 3. 40X magnification.

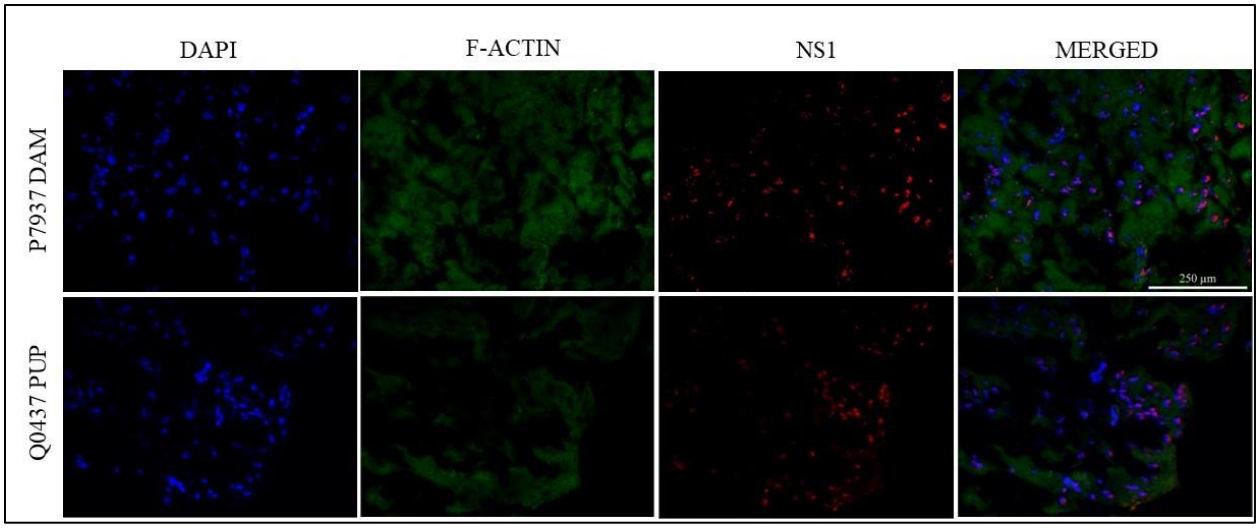


Figure 4.2: Cerebral cortex of P7937 dam and Q0437 pup. The NS1 score for this image of the dam (top) is 4 and the score for this image of the pup (bottom) is 3. 40X magnification.

We euthanized some pups from the same litters at different for ages the purpose of comparing the NS1 levels longitudinally. The pups were aged 8, 22, and 30 weeks, and ANOVA was conducted (**Table 6**) The calculated p-value was 0.00000000255, deeming the difference in NS1 at different ages to be highly significant. NS1 was detected in all pups necropsied at 8 weeks. Little to no NS1 was seen in 22- and 30-week-old pups **(Table 6 and Figure 5).** At 8 weeks the average NS1 score was 3.50, at 22 weeks it was 0.08, and at 30 weeks it was 0.40 ($p<0.0001$) The p-value from Welsh's t-test comparing the 22- and 30-week-old age groups and was 0.06 which makes those two data sets not significantly different from each other (**Figure 5).**

Welsh's t-test comparing the average NS1 scores of the cerebellum and the cerebral cortex of the experimentally inoculated dams and 8-week-old naturally infected pups revealed no statistical significance viral detection in any one of the regions. However, the average NS1 score of the cerebral cortex (3.97) was slightly higher than that of the cerebellum (3.41).

Table 6. Results of ANOVA of NS1 score across different age groups of pups.

SUMMARY				
Groups	Count	Sum	Average	Variance
8 weeks	6	20.79167	3.465278	0.307234
22 weeks	2	0.166667	0.083333	0.013889
30 weeks	11	4.833333	0.439394	0.205682

ANOVA						
Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	39.17547	2	19.58774	86.89064	2.55E-09	3.633723
Within Groups	3.606876	16	0.225429			
Total	42.78235	18				

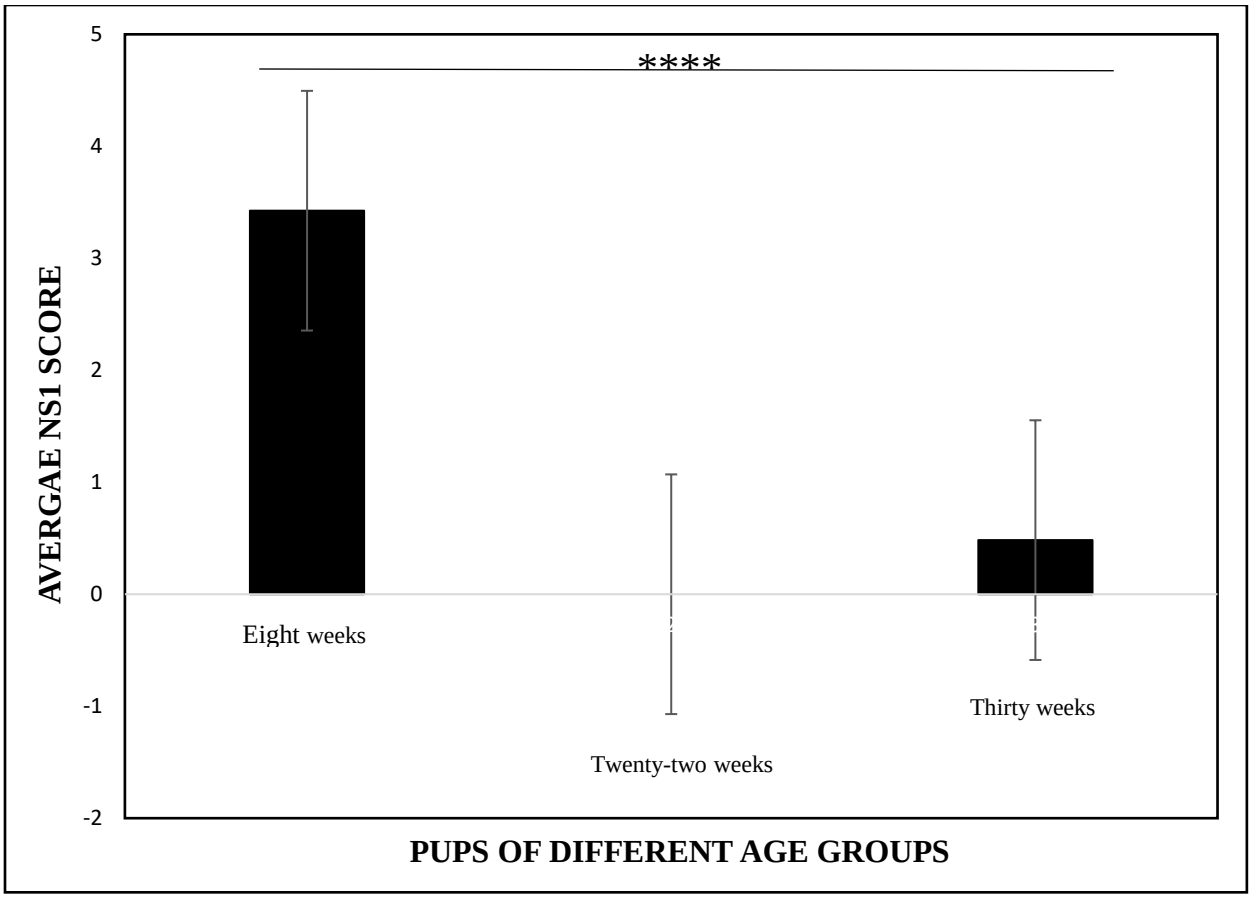


Figure 5: **Comparison of average NS1 scores of the eight pups euthanized at 8 weeks (n=6) of age with NS1 scores of their littermates euthanized at 22 weeks of age (n=2) and 30 weeks of age pups (n=11)**. The average score of 8-week-old pups was 3.47, average score of 22-week-old pups was 0.08, and that of 30-week-old pups was 0.44. **** indicate a p-value of less than 0.0001. The bars represent standard error.

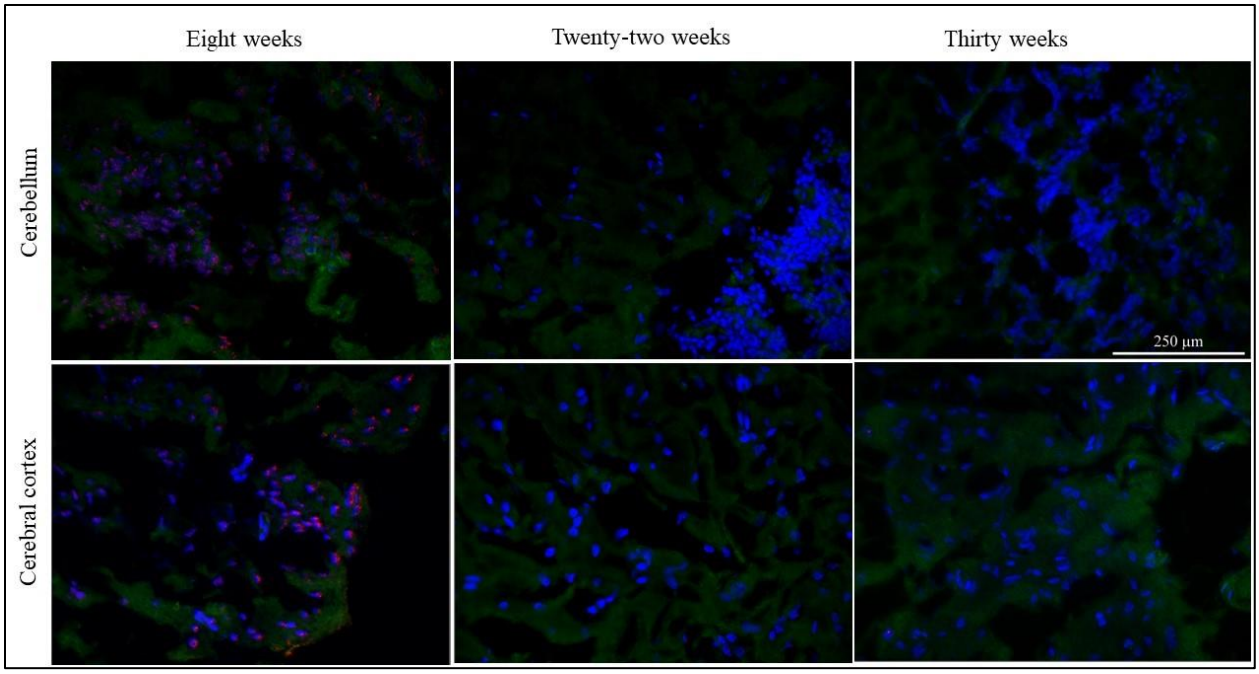


Figure 6: NS1 signals in the cerebellum and cerebral cortex of animals Q0437 (8 weeks), Q0434 (22 weeks), Q0443 (30 weeks). The score in these images for pups of 8 weeks (left) is 4 (top) and 3 (bottom). The score for pups of 22 weeks (middle) is 0 (top) and 0(bottom). The score for pups of 30 weeks (right) is 0 (top) and 0 (bottom). 40X magnification.

CHAPTER V

DISCUSSION

A comparison of IHC results and ELISA titers reveals that there is a similar trend in the IHC results and the ELISA results (**Tables 4 and 5**). A coefficient of correlation 0.72 was calculated from both data sets indicating strong, positive correlation. However, when comparing the dams with only the infected pups (8 weeks at necropsy) the coefficient of correlation 0.62 suggesting a positive association but not necessarily a correlation. NS1 was detected in every animal that was deemed Zika positive or indeterminate by ELISA, and ELISA negative animals had little or no NS1 detection. The correlation between the ELISA titers and the IHC results of the pups implies that antibody levels decrease as viral load decreases, rather than the alternative that viral load would decrease as antibody levels increased and enabled elimination of the virus. Q0444 had an indeterminate ELISA titer of 0.96, viral proteins were still detected when IHC was conducted on the animal and resulted in a score of 3.17. This finding implies that IHC is a more sensitive method in determining viral infection than the ELISA assay and that IHC can be used to determine if an animal is truly infected if it has an indeterminate ELISA titer.

It was interesting, although not unexpected, that NS1 could be detected in the brain, despite intraperitoneal inoculation. The experimentally inoculated dams had a higher NS1

average score than the naturally infected pups, but the difference was not statistically significant ($p=0.497634$). NS1 average scores were also higher in 8- week-old pups than in 22- or 30-week-old pups. ($p<0.0001$) This result suggests that the animals can partially clear the virus from the brain as they grow older. The absence of a developed immune system in neonatal *Monodelphis* may make them vulnerable to viral infection. They obtain antibodies from their mothers along with Zika virus, and, as their immune system develops, they apparently can clear out some of the viral particles. As the viral load is diminished, the antibody titer is decreased. Another explanation for the viral load and viral antibodies levels decreasing is due to the decreasing NPCs. Zika primarily affects the NPCs and as NPCs begin to differentiate the viral proteins are cleared out.

A higher volume of cells was observed in both control and infected animals in any given field of view in the cerebellum as compared to the cerebral cortex. The cerebellum had coils of densely packed cells and space within the coils with sparse cells. The cerebral cortex was uniform in having sparse cells throughout. Research on immune-competent mice has shown Zika viral detection in the cerebral cortex, (Li et al., 2018) and our results are in line with those findings. In fact, a trend of higher abundance of NS1 was seen in the cerebral cortex than in the cerebellum, although the difference was not significant.

A limitation of the *Monodelphis domestica* model is that when pups are born with microencephaly, or any visually apparent malformations, they may not survive (although one such instance of survival of such a pup has been reported (by Thomas et al.,2019). This makes it impossible to study some neural malformations that occur due to or comorbid with Zika virus.

While infinity analyze is a helpful software for taking fluorescent images from a widefield fluorescent microscope, it is incredibly time consuming to try and take an image of the entire tissue. Another limitation is that the NS1 protein is not detectable when the tissue is being viewed at 10x magnification, limiting the efficacy of the tissue screening process. This issue may be resolved by using additional software that can facilitate fluorescent whole slide scanning. This technique would help with scanning the entire tissue to take images that are a better representation of the samples. A third limitation is that the NS1 secondary antibody fluorophore is exceptionally sensitive to light, limiting the amount of time one can observe and image tissues. IHC would also yield better images when imaged using a confocal microscope (which was not available for this study) rather than a widefield fluorescence microscope.

The existence of naturally infected pups opens a world of questions to be explored. Since, the *Monodelphis domestica* pup brain samples for this study did not have any apparent anatomical malformations, it would be interesting to conduct behavioral studies on naturally infected pups, to observe if infection at that early developmental stage impairs learning, memory, or any other cognitive abilities later in life. For instance, maze experiments could reveal how long these pups take to reach their destination in comparison to uninfected pups. Further research should also be done in detecting NS1 in coronal sections of the brain to enable a more in-depth understanding of where the viral proteins are localized. IHC on different organs like the spleen, placental tissue, and reproductive organs in comparison to brain tissue could also be done to track the pathogenesis of Zika in naturally infected pups. It may also be interesting to stain tissues with NeuN, a neuronal marker, and Iba, which is a glial cell marker, along with the NS1 marker to the predominant cellular location of the virus. Nestin, which is neural stem cell

biomarker, could also be used to determine the localization of NS1 in neural progenitor cells of the pups.

The major conclusions of this study are as follows:

1. IHC results and ELISA results have a strong, positive correlation.

2. IHC can confirm that an animal is infected when the ELISA result of the animal is in the indeterminate range.

3. The viral protein concentration of experimentally inoculated dams naturally infected pups is statistically not significant, and therefore similar.

4. When experimentally inoculated dams give birth to naturally infected pups the viral protein concentrations in the brains of the pups diminish over time, at least after 8 weeks of age.

5. Pups euthanized at 8-weeks-of-age had a higher viral protein concentration in their brains than their siblings that were euthanized at 22 and 30 weeks of age.

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BIOGRAPHICAL SKETCH

Mohini Moulick earned her Bachelor of Arts degree in biology from Concordia University Irvine (CUI) in May of 2020. During that time, she worked in CUI's microbiology and marine laboratory. She earned her M.S. in Biology Program at The University of Texas Rio Grande Valley (UTRGV) in August 2023. During her time in the graduate program, she also served as a teaching assistant for the general biology and marine ecology instructional lab. Additionally, Mohini was employed by Gladys Porter Zoo as an aquarist and by UTRGV as a biology tutor for veteran students. Mohini aspires to earn a Ph.D. in either neuroscience or virology and to do biomedical research in industry to help in the advancement of health science. Mohini can be contacted via email (mohini.moulick01@gmail.com)