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ASSOCIATION OF TRIM GENE VARIATION AND LPDV INFECTION PATTERNS IN WILD TURKEYS

By

Bennett Blaine Fry

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford, MS

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Approved By

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ABSRACT

BENNETT BLAINE FRY: Association of TRIM Gene Variation and LPDV Infection Patterns in Wild Turkeys
(Under the direction of Richard Buchholz)

Emerging infectious diseases pose a serious threat to various species throughout the globe, including humans. The lymphoproliferative disease virus (LPDV) in wild turkeys is an example of disease virus whose impact on its host requires additional study. Although the first description of lymphoproliferative disease came from outbreaks in domestic turkey flocks in Europe, it is now known that LPDV is widespread in wild turkey populations in North America. In an effort to understand what may affect an individual turkey's susceptibility to this virus, variation in part of the anti-viral TRIM62 gene was investigated. DNA was extracted from hunter-collected, turkey blood samples and then subjected to PCR to amplify the TRIM62 gene fragment and to test for LPDV infection. The TRIM62 and LPDV amplicons were sequenced commercially .The occurrence of LPDV positive samples from wild turkeys collected from three different states was 50%. Phylogenetic analysis of the LPDV sequences showed patterns of genetic distance among collected samples and LPDV sequences available in GenBank that suggest that historical restoration efforts may have dispersed the virus over great geographic distance. In the exon 3 portion of the TRIM62 gene examined, the only sequence variation observed were two separate point variants that would not have resulted in a different peptide sequence compared to the consensus sequence. Therefore, these variants could not affect LPDV infection patterns among individuals. Additional research on other exons of TRIM62 and other TRIM genes and their association with LPDV infection is warranted.

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Introduction

Plants and animals alike are plagued by a wide variety of parasites that rely on host organisms for the resources necessary for survival and reproduction. These parasites include microscopic organisms such as bacteria, fungi, and viruses and macroscopic ones like ticks and tapeworms. Although parasites usually do not directly kill their hosts, parasitic infection can decrease host fitness and increase vulnerability to natural causes of death such as predation and starvation (Marcogliese & Pietrock 2010). Infection is a threat to the survival and reproduction of the host, but organisms are not completely defenseless. Host organisms have developed ways of preventing or limiting the extent of infection by commonly encountered pathogens. The evolutionary arms race between pathogens and their hosts has led to the evolution of sophisticated immune defenses in vertebrate hosts and selection on pathogens to evade those host defenses. When disease spreads to new host species, however, the pathogens may have an advantage over the naïve host's immune defenses.

Novel pathogens and emerging diseases severely impact numerous species including humans. This has become a subject of increasing concern with populations encountering pathogens that were previously noninfectious to humans. In recent decades, multiple animal coronaviruses (including MERS-CoV, SARS-CoV, and SARS-CoV-2) have evolved to become infectious to humans. (Centers for Disease Control and Prevention 2020). Without having previous exposure to these viruses, human populations are susceptible to severe outbreaks of deadly disease.

The seriousness of novel diseases in wildlife species is exemplified by bighorn sheep pneumonia. According to the National Park Service, in the western United States, populations of bighorn sheep (*Ovis canadensis*) have been critically affected by a variety of novel bacteria (including *Mannheimia haemolytica, Pasteurella trehalosi, Pasteurella multocida,* and *Mycoplasma* spp.) transmitted from domestic herds of goats (*Capra aegagrus hircus*) and sheep (*Ovis aries*). Because the bighorns did not evolve with these pathogens as the domestic goats and sheep did, the wild sheep suffer high mortality across all ages (National Park Service 2018).

Bacteria are not the only organisms that may pose a threat as novel pathogens to wildlife. The chytrid fungus (*Batrachochytrium dendrobatidis*) is associated with worldwide decline of amphibian species (University of California Riverside Center for Invasive Species Research 2021). External growth of the fungus occurs on keratinized skin of the amphibian and eventually causes death in susceptible species. Chytrid fungus outbreaks were first described in 1998 in Australia, but currently infections occur on nearly every continent where some amphibian species are experiencing dramatic population declines. For example, during the span of 4 months in 2004, an 80% reduction in density and a 60% reduction in amphibian diversity were recorded with a 60% increase of chytrid fungus prevalence in Panama. As seen with the bighorn sheep, spread of this pathogen to species that did not evolve with it is having a major impact on their populations.

Another wildlife species that may be experiencing novel threats is the wild turkey (*Meleagris gallopavo*). Although American conservation efforts helped re-establish healthy numbers of wild turkeys after the species was extirpated from much of its geographic range, recent population declines are causing concern. The National Wild Turkey Federation reports that the United States' wild turkey population has fallen nearly 15% over the last decade from an

all-time high of 6.7 million to 6.2 million (Perrotte 2021). Several states have seen a decrease in harvest numbers believed to be a result of smaller populations (Casalena et al. 2007; Hughes et al. 2007; Tapley et al. 2011). Butler and Godwin (2017) described and investigated a decrease in Mississippi's wild turkey population from 2012 to 2016. Among the new threats that may be impacting wild turkey populations and causing such declines is the oncogenic retrovirus Lymphoproliferative Disease Virus (LPDV).

In my thesis, I first introduce the reader to the LPDV virus, discuss basic characteristics of retrovirus structure and lifecycle, and describe the importance of TRIM genes in the innate protections against viral infections of vertebrate hosts. Finally, I describe an investigation of nucleotide sequence variation in a part of the TRIM62 gene of wild turkeys and test its association with the occurrence of LPDV infection of individual birds.

Background Information

Previous studies of LPDV

Lymphoproliferative disease virus (LPDV) is an avian retrovirus recently discovered in wild turkeys in North America that previously was thought to be restricted to domestic turkey flocks in Europe and Israel (Allison et al. 2014). In one study, 564 out of 1,164 samples (47%) from hunter-harvested wild turkeys were positive for LPDV proviral DNA. The investigation spanned 417 counties in 17 states. LPDV was found to be prevalent in all studied states, but birds in the Northeast were most affected having the highest infection percentage (Thomas et al. 2015). A more recent study showed that 40% of hunter harvested samples from three different counties in Mississippi contained LPDV proviral DNA (Hyche 2019).

Phylogenetic analysis of fragments of the LPDV genome collected from 185 wild turkeys revealed that there are two major clades of the virus within the United States with one having a much lower frequency (Thomas et al. 2015). The lower frequency clade contains the original Israeli prototype strain that was first recovered in 1978. This shows a direct relationship between the viral strains in Europe and those in North America. The more numerous clade contains members that belong to a single North American lineage. These viral strains are slightly isolated by state in the Eastern United States, but evidence of viral gene flow is present (Thomas et al. 2015).

In contrast to the earlier studies that focused on a more national distribution of LPDV (Allison et al. 2014; Thomas et al. 2015), Alger et al. (2017) focus on birds from a single US state to investigate possible risk factors for LPDV infection in wild turkeys. Bone marrow was collected from hunter-harvested birds from 2012 to 2014 in New York state during fall hunting seasons. The fall season includes both males and females, whereas the spring season only include males. The bone marrow samples were used to test for LPDV DNA using PCR primers, and the infection percentage was 55%. The authors determined that age and sex were strong predictors for LPDV infection. Adult birds were more likely to be infected than juveniles, and a higher percentage of females experienced infection than males. Habitat was also determined to be a risk factor with turkeys sampled from lands with a higher ratio of agricultural land to forest showing a greater chance of LPDV infection. They hypothesize that this may be due to greater viral transmission among turkeys in large gatherings where spilled grain remains after crop harvest.

An even more recent study has shown that LPDV is prevalent among wild turkeys in Canada. MacDonald et al. (2019) described the first detection of LPDV in Ontario, Canada and found that 65% of tested birds were infected with the virus. Wild turkeys have been reintroduced

to Ontario using birds from Missouri, Iowa, Michigan, New York, Vermont, New Jersey, Michigan, and Tennessee. Phylogenetic analysis of the viral samples revealed that the Ontario strains were included within the North American clade that is separate from the Israeli clade. This provides evidence that viral strains now circulating in Canada may have been brought from the eastern United States. This study also tested for other infections including avian reticuloendotheliosis virus (REV). REV is similar to LPDV in that it can cause lesions of irregular lymphoid cells, and 4% of birds were found to be co-infected with both viruses (MacDonald et al. 2019).

The only other known host of LPDV is the domestic chicken (*Gallus gallus*), suggesting that this virus is restricted to birds in the order Galliformes (Cornell University 2018). At this point, wild turkeys are the only free-living species known to be infected by the virus. Although the virus infects wild turkeys throughout the United States, external symptoms in these birds seem to be rare. Out of 74 hunter-harvested samples collected in South Carolina, 44.6% of them tested positive for LPDV proviral DNA, but none of the birds were reported to display externally visible signs of disease (Allison et al. 2014).

Host pathology from oncogenic LPDV is the result of cancerous cell changes. This virus is commonly associated with tumors in the organs of infected individuals, and the organs most affected by the virus include the spleen, pancreas, and liver (Cornell University 2018). The most common characteristic of the disease in domestic turkeys is an enlarged spleen. Lesions of pleomorphic lymphoid cells were observed in the spleen as well as other organs, and most infected individuals did not display signs of infection until death (Biggs et al. 1978).

In wild turkeys, possible external signs of LPDV infection include skin nodules on the head, neck, and feet. Mode of viral transmission to new hosts and persistence of the infection is unknown for wild turkeys (Cornell University 2018).

LPDV may not be the only retrovirus causing neoplasia in wild turkeys. Niedringhaus et al. (2019) conducted a large-scale investigation of neoplasia in wild turkeys via retroactive examination of preserved specimens and diagnostic records submitted to the Southeastern Cooperative Wildlife Disease Study from 1980 to 2017. A total of 851 birds were examined and neoplasia was documented in 59 of them (6.9%). Out of the neoplasia cases, 59% were associated with LPDV, 26% were associated with reticuloendotheliosis virus (REV), and 8% were associated with both viruses. As seen in previous studies, the most common symptoms of the sick birds that were submitted were emaciation, skin nodules, and organ lesions. Nodules were examined microscopically showing pleomorphic lymphoid cells in all but two cases. One case appeared to have irregular myeloid cells and the other showed spindle cells being affected. This study also shows that pathological changes in wild turkeys are nearly the same as those that have been described in domestic turkeys. In the next section of this review, I describe the group of viruses to which LPDV belongs and their basic lifecycle.

Biology of retroviruses

Retroviruses have long been described as the causative agents of disease in many vertebrate species. These viruses can be divided into three subfamilies (Oncoviruses, Lentiviruses, and Spumaviruses) based on morphological and genetic features determined by electron microscopy and nucleotide sequencing, respectively. Retroviruses can be further differentiated into A-type, B-type, C-type, and D-type by morphological differences. C-type retroviruses have an electron-dense core and most oncoviruses are of this variety (Cloyd 1996). LPDV is a type-C retrovirus;

therefore, it is not surprising infected individuals may have organ tumors and unnatural skin growths (Cornell University 2018).

The infectious form of a virus is called a virion. The virion is composed of a protein covering, called the capsid, which encloses the nucleic acid of the virus. Virions may also acquire an envelope from the host cell membrane when budding off from the cell. The enveloped virion of a retrovirus contains a diploid RNA genome and an inactive reverse transcriptase enzyme that becomes activated after penetration of the host cell and uncoating of the viral RNA. The RNA genome of all retroviruses contains the genes *gag*, *pol*, and *env*. Other genes may be present within the genome depending on the specific virus. The *gag* gene encodes structural proteins used for assembly of the capsid. The *pol* gene encodes reverse transcriptase, and *env* codes for envelope proteins (Weiss 1996). Penetration occurs when the envelope proteins interact with receptors on host cells causing the cell to take in the virion via endocytosis. Once inside the cell, the structural components of the virion break down to release the viral RNA (uncoating). The reverse transcriptase then converts the RNA genome into DNA which is now known as the provirus. The provirus is integrated into host DNA by another viral enzyme, integrase, that is produced from part of the *pol* gene. Once contained within host chromosomal DNA, the provirus may remain latent with no viral proteins being made, or viral expression may occur (Weiss 1996). If the provirus is expressed, mRNA is transcribed that will be used for the translation of viral proteins, and also will be used for the RNA genome of new virions. Since it is continuous with host chromosomal DNA, transcription of the provirus is mediated by normal cellular transcriptional regulators (Weiss 1996). In order for a mature virion to be assembled, the gag protein must be cleaved into the major structural proteins by a viral protease, and the envelope proteins must be synthesized. The envelope proteins are made in the rough

endoplasmic reticulum, processed in the Golgi apparatus, and sent to the cell surface for incorporation into the new, budding virion. (Weiss 1996). Once all of the viral proteins and genomic RNA are present, a new virion may be assembled and bud off from the host cell to infect others.

Retroviruses have been documented to be transmitted in various ways including vertically, horizontally, and genetically. Vertical transmission involves the virus spreading from mother to offspring. Horizontal transmission occurs when the virus is spread between individuals in close contact. For a retrovirus to be transmitted genetically, the provirus must be integrated within germline cells and passed on to the zygote via the sperm or egg (Weiss 1996). Currently, the path of transmission of LPDV in wild populations is unknown; however, it has been shown, experimentally, that the virus is easily spread via horizontal transmission between in-contact turkey poults (McDougall et al. 1978, in Thomas et al. 2015).

As described, there are many steps in the life cycle of a retrovirus, and interference at any of these could potentially have detrimental effects on replication. Because of this, vertebrate immune responses have evolved mechanisms to prevent completion of the retroviral life cycle.

TRIM proteins structure and function

Vital components to vertebrate immune response are tripartite motif (TRIM) proteins. In humans, TRIM proteins are a family of over 80 E3-ubiquitin ligases involved in many cellular processes. Depending on genome size, other species may have a greater or lesser number of TRIM proteins (Gent et al. 2018). According to Tol et al. (2017), TRIM proteins are named as such because of the three-part RBCC domain conserved among them. The “R” in RBCC represents a “really interesting new gene” (RING) E3 ligase domain. The “B” represents one or

two B-box domains, and the "CC" comes from the coiled-coil domain. TRIMs may also contain certain C-terminal domains with the most common being the PRY and SPRY domains. The ligase activity of TRIM proteins is crucial to viral immune defense. There are three different kinds of enzymes involved in post-translational ubiquitin modifications of proteins: E1, E2, and E3-ubiquitin enzymes (Hage & Rajsbaum 2019). E1s are activating enzymes that allow recruitment of other enzymes when bound to ubiquitin. Ubiquitin is then passed to the E2 conjugating enzymes that aid in determining the arrangement of ubiquitin molecules that will be added to a protein. Lastly, E3 ligases interact with the E2s in order to determine the specific proteins that will be modified and also to transfer the ubiquitin from the E2s to the target proteins (Tol et al. 2017).

Ubiquitin itself is a unique molecule that is involved in many immune responses. Composed of 76 amino acids, ubiquitin is capable of associating with itself and other proteins either covalently or noncovalently (Tol et al. 2017). After the addition of ubiquitin to other proteins has been catalyzed by the three ubiquitin enzymes, it can mediate different responses within a cell. Some of these include the localization of the proteins, signal transduction, movement of the proteins, protein stability, and how the cell proceeds through the cell cycle (Tol et al. 2017). When ubiquitin is covalently attached to another protein, it is usually done at one of the lysine amino acids within ubiquitin. Ubiquitin contains seven of these at positions K6, 11, 27, 29, 33, 48, and 63. These different lysine amino acids allow for various conformations of ubiquitin modifications which determines the cellular response (Tol et al. 2017).

Although there are many effects on proteins caused by ubiquitin modification, two well-studied mechanisms are K48 and K63-linked ubiquitin chains. An ubiquitin chain linked via K48 usually marks the protein for proteasomal degradation and recycles its component amino acids

(Tol et al. 2017).On the other hand, ubiquitin chains linked via K63 play a quite different role inside cells. This often causes the protein to be involved in the transduction of cell signaling (Tol et al. 2017). These examples do not include all possibilities of cellular responses to these modifications but common ones.

Involvement in viral immunity

The antiviral response of hosts to viral infection recognizes potential pathogens, prevents infection and replication of the pathogens, and also eliminates them. TRIM proteins are involved in many antiviral strategies that can be summed up into three main classes (Figure 1). These classes are modification of immune response signaling, direct restriction of viruses, and autophagy mediation (Gent et al. 2018).

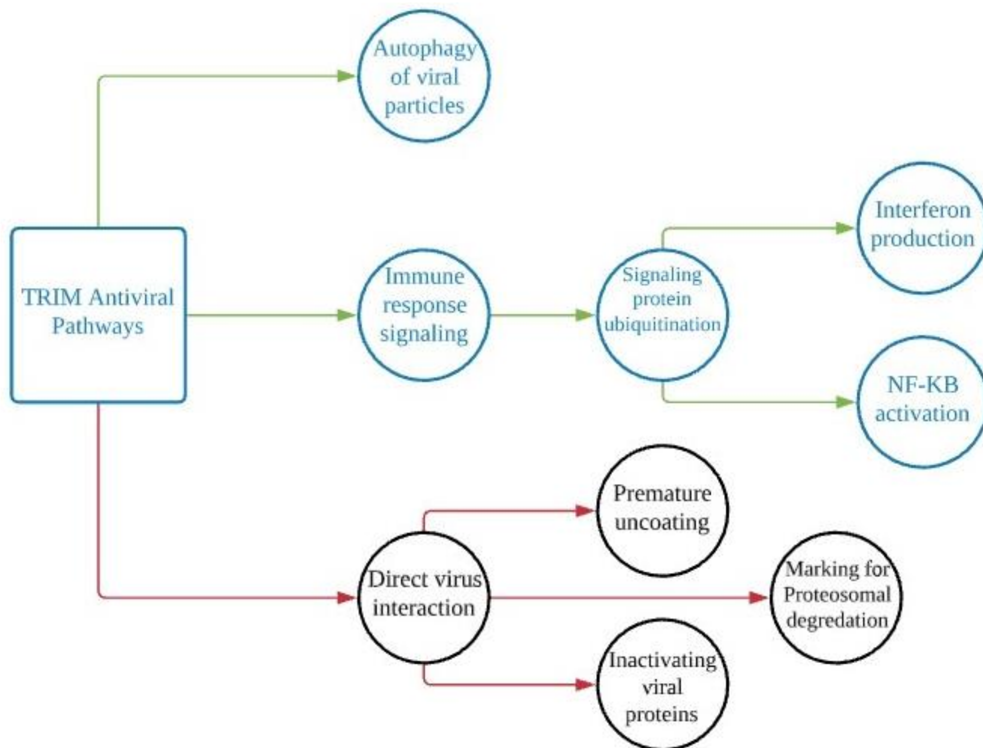


Figure 1: A summary of the antiviral strategies used by TRIM proteins is shown. The green arrows represent TRIM proteins causing an increase in cellular activity, whereas the red arrows show a reduction in viral activity.

Cells can determine the presence of pathogens by detecting pathogen-associated molecular patterns (PAMPs). PAMPs have certain characteristics that mark them as foreign when detected by pattern recognition receptors (PRRs) within host cells (Tol et al. 2017). Upon stimulation by PAMPs, PRRs initiate a cascade of signaling pathways that mediate viral immune responses. (Hage & Rajsbaum 2019). Because the transduction of this signaling cascade relies on post-translational modifications of proteins, ubiquitination being one, TRIM proteins are heavily involved in the control of immune signaling (Hage & Rajsbaum 2019). Two crucial instances of this control would be the regulation of interferon induction and the activation of NF-κB. (Tol et al. 2017). Simply put, interferons are molecules released by infected cells that warn the cellular immune system of viral infection and also induce an antiviral state in nearby, uninfected cells. This antiviral state includes the production of hundreds of viral defense proteins that are otherwise unnecessary (Goodsell 2010). NF-κB, on the other hand, is a transcription factor that regulates gene expression of various components of the immune system (Moynagh 2005). TRIM proteins are capable of regulating these signaling pathways at various points due to their ubiquitin ligase activity. Tol et al. (2017) state that TRIM signaling control can occur between the time when PAMPs have been recognized by PRRs to the regulation of transcription factors for antiviral genes. This also can occur from the time signaling complexes are assembled to degradation of inhibitors of antiviral genes.

TRIM proteins also employ strategies to directly interact with viruses and restrict viral replication. Some TRIMs bind to the virions themselves and cause premature uncoating. After uncoating, the structural components of the virion are released as PAMPs into the cell cytoplasm and have a greater chance to be recognized by PRRs thus amplifying the immune response. Other TRIMs can prevent viral proteins from performing their normal functions by directly

binding to them (Gent et al. 2018). TRIM proteins also play a role in proteasomal degradation. As mentioned earlier, a protein marked by K48-linked ubiquitin will usually be degraded by a proteasome. This process is helpful in removing unwanted materials from the cell such as viral particles (Hage & Rajsbaum 2019). Certain TRIM proteins are able to signal for viral proteins to be degraded via K48 linkage of ubiquitin. For instance, in humans, TRIM5α targets the viral NS2B/3 protease for proteasomal degradation which in turn inhibits replication of TBEV (Sofjin strain), Kyasanur Forest disease virus (KFDV), and LGTV (Hage 2019).

Another form of immune response that involves TRIM proteins is autophagy mediation. Autophagy is a cellular process that leads to the destruction of damaged organelles, unnecessary proteins, and even viral particles. This occurs when the unwanted material is enclosed by a double-membrane (autophagosome) and then fuses with a lysosome. In recent studies, TRIM proteins have been shown to be important regulators of various types of autophagy including that of viral particles. Some TRIMs regulate the different components of autophagy, whereas others act as cargo receptors that are highly specific to certain viruses. For example, upon HIV-1 infection, the TRIM5α protein recognizes the capsid and recruits the machinery necessary for autophagy while restraining the viral cargo (Gent et al. 2018). Similar to proteasomal degradation, destruction of viruses via autophagy can release PAMPs and amplify the immune response.

Viral antagonism and hijacking of TRIM proteins

During the co-evolutionary battle between host and parasite, viral pathogens have evolved means of evading host immune responses including antagonism and hijacking of TRIM proteins. Viral antagonism of TRIM proteins alters many processes that ultimately lead to an insufficient immune response and successful viral infection. Viruses have the ability to interact with TRIM

proteins either directly or indirectly to prohibit them from carrying out their immune functions. Direct interaction would include viral products designed to target TRIM proteins so that they are unable to associate with other proteins. Indirect interactions can interrupt signaling pathways that are mediated by TRIM proteins (Tol et al. 2017). Some viruses are also able to take advantage of TRIM functions that can promote viral replication. Hijacked TRIM proteins can increase the activity of viral proteins and affect signaling pathways so the environment within the cell is more suitable for viral replication (Gent et al. 2018). An excellent example of this can be seen with the Japanese encephalitis virus in humans. Upon infection, the virus induces a higher expression of TRIM21. This higher expression in turn downregulates an interferon signaling pathway and reduces the antiviral response (Gent et al. 2018).

TRIM gene variation

Interspecific comparisons of the TRIM genes suggest that the function of the proteins produced from these genes has been evolutionary conserved across vertebrates (Boudinot et al. 2011). For example, among the zebrafish (*Danio rerio*) and the spotted green pufferfish (*Tetraodon nigroviridis*) the nucleotide sequences of many TRIM genes were conserved between the two fish species, and with mammals. Similar expression patterns of the TRIM proteins within tissues were seen as well, suggesting that the function of TRIM genes has been conserved across many species. Certain regulatory sequences of TRIM genes have also persisted throughout millions of years of primate evolution (He et al. 2016). Nevertheless, there is some evidence of intraspecific variation in specific TRIM genes that supports the notion that they remain under directional selection.

Differences in the genes that code for TRIM proteins may affect how they are formed and what functions they are able to carry out. Ovsyannikova et al. (2013) studied differences in

measles vaccine immunity in relation to polymorphisms in TRIM genes. In this study, 764 subjects from ages 11 to 22 were given two age-appropriate doses of the measles vaccine. The results showed that polymorphisms in human TRIM5, TRIM22 and TRIM25 are associated with differences in the adaptive immune responses (antibody titer, cytokines, and the IFN-γ Elispot response) to the measles vaccine.

Lab experiments have even tested the effects of the absence of entire TRIM genes. Vaysburd et al. (2013) demonstrated that the immune systems of mice lacking TRIM21 were unable to defend against non-lethal doses of mouse adenovirus 1. In contrast, TRIM6-absent cells from the A549 cell line purchased from the American Type Culture Collection cleared Ebola virus more effectively than the wild type cells that contained the TRIM6 gene (Bharaj et al. 2017). Although these *in vitro* studies and investigations of model laboratory species have elucidated the wide variety of functions of TRIM proteins during viral infection, limited information is available about TRIM gene variation and its effects in bird species.

Li et al. (2019) investigated the role of TRIM62 in restricting Avian Leukosis Virus Subgroup J (ALV-J), a virus closely related to LPDV. Both viruses, along with rous sarcoma virus (RSV), are included within the clade *Alpharetrovirus* (Allison et al. 2014). The results from Li et al. (2019) show that deletion of the TRIM62 SPRY domain nearly renders the protein ineffective. If the SPRY domain is present, the antiviral efficiency is then dependent upon the other domains. This study also showed that overexpression of TRIM 62 in chicken embryo fibroblasts (CEFs) greatly restricted viral replication, whereas under-expression caused viral replication to increase. Deficiency studies such as this suggest that inter-individual variation in TRIM gene sequences could affect host susceptibility to viruses.

The first study to have identified polymorphisms in the TRIM subregion of the Japanese Quail (*Coturnix japonica*) genome described allelic diversity in TRIM and closely related genes. PCR products and nucleotide sequencing of samples from 321 individuals (11 quail strains) were used to identify multiple TRIM gene alleles, and it was determined that recombination was common in the TRIM genomic subregion (Suzuki et al. 2012). Although the functions of TRIM proteins are conserved across vertebrate species, the coding sequences of TRIM proteins may vary. There do not seem to be any more recent investigations inspecting TRIM gene variation of avian species. The wild turkey TRIM62 protein lacks the SPRY domain of the chicken (Figure 2), suggesting that perhaps turkeys will show greater susceptibility to this group of viruses just as the SPRY-deficient chicken cells did in the study of Li et al. (2019). It would be interesting to see how differences in the nucleotide sequence of individuals of this wild species for the remaining TRIM62 protein domains relate to immune function and viral susceptibility.

```
10         20         30         40         50
MACSLKDELL CSICLSIYQD PVSFGCEHYF CRRCITEHWV RQEPQGARDC
        60         70         80         90        100
PECRRTFTEP TLAPSLKLAN IVERYSAFPL DAILGAQRSP FPCKDHEKVK
       110        120        130        140        150
LFCLTDRTVV CFFCDEPAMH EQHQVTNVDD AFEELQRELK EQLQGLQESE
       160        170
RGHTEALHLL KRQLADTKVG AR
```

Figure 2: The 172 amino acid long wild turkey TRIM62 protein has only three domains, lacking the SPRY domain shown by Li et al. (2019) to be essential to anti-retroviral outcomes in cells of the domestic fowl. Domain amino acid sequences as are color-coded as: Yellow (RING-type domain, 44 amino acids), Green (B box-type, 41 amino acids), and Red (Coiled coil, 21 amino acids). Based on predicted annotation at https://www.uniprot.org/uniprot/G1N5A7 .

Hyche's (2019) previous work on LPDV infection in hunter-killed male wild turkeys demonstrated that more than half the individuals sampled were not infected. I hypothesize that these differences in LPDV infection in wild turkeys is affected by TRIM gene differences among individuals. Wild turkeys were extirpated throughout much of their geographic range by the

1940's (Leberg 1991). Initially, restoration of wild turkey populations to their former range relied on translocated wild-caught birds from states that had protected the birds (Tapley et al. 2007). As the populations founded by translocated birds grew, they were used to establish additional populations within the same state (Mock et al. 2002). As a result of this genetic bottleneck, it is suspected that some wild turkey populations may lack genetic variation. Theoretically, genetic homogeneity in host populations may make them more susceptible to disease threats, such as LPDV.

Objectives

The primary objective of my research is to investigate the sequence variation in the TRIM62 gene of wild turkeys and determine if the LPDV status of those birds is associated with TRIM gene variation. A second goal is to describe the similarity of the LPDV samples in my study to the nucleotide sequences available from previous research done in the USA and Canada.

Methods

Sample collection

Blood samples collected by volunteers from hunter-harvested wild turkeys in Mississippi, Tennessee, and Oklahoma. During the 2018, 2019, and 2020 wild turkey hunting seasons, sampling kits were distributed to hunters including an instruction sheet, data sheet, pencil, plastic bulb pipette, and a numbered GeneMate 2 mL screwcap tube (Part number C-3318-2) with 1 mL of Fisher 100% ethyl alcohol (product no. BP2818-500). After harvesting a bird, hunters were instructed to transfer one drop of fresh, uncoagulated blood using the pipette to the screwcap tube with ethanol. Body measurements and other information were recorded on the included data sheet (see Hyche 2019). Blood samples were moved to a refrigerator or freezer as soon as possible, and then stored at -20°C once collected from the hunters. Alger et al. (2015)

demonstrated that PCR can be used to amplify and detect LPDV DNA extracted from whole blood as accurately as from other tissues that were harder to sample and process.

DNA extraction

DNA was extracted from the blood samples using different methods depending on year of collection. A detailed explanation of DNA extraction for the 2018 and 2019 samples is given by Hyche (2019). In November of 2020, DNA extraction of that year's samples was done using the Macherey-Nagel NucleoSpin® Tissue kit (REF 740952.50) . Before following the kit's instructions, the samples were vortexed to resuspend the cells, and 80 µL of the blood-ethanol mixture was pelleted in 1.5 mL microcentrifuge tubes using an Eppendorf Centrifuge (model no. 5415C). To remove excess ethanol, the pellet was resuspended in sterile water, centrifuged again to form a pellet, and then the supernatant of water and ethanol was removed. DNA was then extracted from the rinsed pellet following the manufacturer's protocol for working with blood samples. Success of the DNA extraction was assessed by comparing the DNA fragment size and amount to a Biotium Ready-to-Use 100 bp DNA Ladder (cat. no. 31032). An Alpha Innotech gel ultraviolet imager (model: AlphaImager® HP) was used to visualize the fragments separated on a 1.5% agaraose gel subjected to 100 V for 45 minutes. The nucleic acid stain used was Biotium GelRed® Nucleic Acid Gel Stain (cat. no. 41003).

LPDV PCR amplification

DNA samples were tested for LPDV infection by PCR with one or two LPDV specific primers. First a primer designed by Allison et al. (2014) that targets parts of the p31 and capsid (CA) *gag* genes of the viral genome was used on all samples. The forward primer sequence is 5'-ATGAGGACTTAC-3', and the reverse primer sequence is 5'-TGATGGCGTCAGGGCTATTTG-3'. For samples that tested negative with the primers

designed by Allison et al., a second primer pair was used. The sequence for this primer pair was provided by Dr. Randal Renshaw of the Department of Population Medicine and Diagnostic Sciences at Cornell University. According to Dr. Renshaw (personal communication with W. Hyche), the p31/CA primers were not identifying all cases of LPDV infection. He suggested using an unplublished primer pair that would target part of the LTR and Matrix *gag* gene that is conserved among different strains of the virus. The forward and reverse primer sequences are 5'-GGGCACGGGATTGGCTT-3' and 5'- AAACGCTCAATACACGACACAAC-3' respectively. The PCR reagents were added to autoclaved VWR 0.2 ml PCR tubes (product no. 20170-004) on ice in the following order: 9.5 µL of nuclease free water, 12.5 µL of GoTaq® Green Master Mix, 0.5 µL of the forward primer at 10 µM concentration, 0.5 µL of the reverse primer at 10 µM concentration, and 2 µL of wild turkey DNA solution. The total reaction volume was 25 µL. The negative PCR control, used to assess contamination, had the same PCR reagents, but a greater volume (11.5 µL) of nuclease free water was used to achieve the total reaction volume without the DNA component. DNA samples known to be infected from prior testing were used as a positive control to ensure that all reagents and the thermal cycler were working correctly. The PCR conditions were programmed into a Hangzhou Bioer Technology Co. Ltd. GeneExplorer Thermal Cycler (model no. GE-96G). This included 1 cycle of 3 minutes at 95°C, 34 cycles of 95°C for 30 seconds; 54°C for 30 seconds; and 68°C for 1 minute, and 1 cycle of 68°C for 5 minutes. The thermal cycler was programmed to hold the tubes at 4°C once the reaction was complete. Following PCR, 5 µL of the product from each sample was used in gel electrophoresis along with 10 µL of Biotium Ready-to-Use 100 bp DNA Ladder (cat. no. 31032) to look for amplification by the primer pair. Samples showing a single band of approximately 400 bp were interpreted as being positive for LPDV infection.

TRIM62 fragment PCR amplification

The TRIM62 gene, LOC104914205, is located on chromosome 23 in wild turkeys and contains 4 exons. The end of exon 2, all of exon 3, and the beginning of exon 4 were targeted for amplification and sequencing in this study (Figure 3). I also report on unsuccessful attempts to amplify other regions of this gene in a manner suitable for sequencing (Table 2).

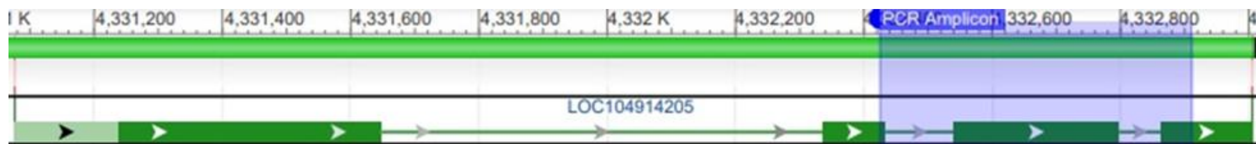


Figure 3: A portion of chromosome 23 in wild turkeys is represented with emphasis on LOC104914205, the TRIM62 gene. The dark green rectangles represent exons 1-4, and the section that should be amplified by these PCR primers is shown in blue and labeled PCR amplicon.

PCR was performed with primer pairs designed using NCBI's Primer BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) against a reference sequence from the domestic turkey (NCBI Reference Sequence: NC_015033.2). The forward primer sequence is 5'-ACACCAAGGTAGGAGCGAG-3', and the reverse primer sequence is 5'-GAAGTCTTCGTAGGTCAGGTTG-3'. The primer pair was designed to amplify from nucleotide position 4332426 to 4332909 of the reference sequence. The PCR for the TRIM62 gene contained the same reagents, but with different primers than for LPDV, as explained previously. PCR conditions for the TRIM62 fragment amplification were: 1 cycle of 95°C for 3 minutes, 35 cycles of 95°C for 30 seconds; 56.5°C for 30 seconds; and 72°C for 1 minute, and 1 cycle of 72°C for 5 minutes. The thermal cycler was programmed to hold the tubes at 4°C once the reaction was complete. Gel electrophoresis was used to test the success of the PCR by looking for an amplicon of the expected product length (484 bp).

Sequencing and genotype determination

LPDV and TRIM62 amplicons were Sanger sequenced for one strand by GENEWIZ (South Plainfield, New Jersey, USA) using the forward primer designed by Allison et al. (2014), the forward primer recommended by Dr. Renshaw, and the forward primer for the TRIM62 gene fragment. Before sending to GENEWIZ, all PCR products were enzymatically treated with Applied Biosystems ExoSAP-IT™ PCR Product Cleanup Reagent (REF 78200.200.UL) following the manufacturer's protocol to remove single and short-length nucleotides that might interfere with sequencing.

The sequences provided by GENEWIZ were analyzed as follows: After automated trimming of low quality ends these TRIM62 amplicon sequences were aligned to the reference genome using the software Geneious Prime 2020.2.4 (Biomatters Ltd) and inspected for sequence variation by Dr. Richard Buchholz. LPDV *gag* amplicon sequences were also trimmed and then were aligned in this software along with 352 similar sequences previous authors had deposited in GenBank (downloaded from NCBI Virus at www.ncbi.nlm.hih.gov). Default parameters were used to allow Geneious' Tree Builder function to construct an unrooted phylogeny using neighbor-joining, genetic distance methods. Based on this phylogeny the single, nearest phylogenetic neighbor to each of the new samples were selected by Dr. Buchholz from among the samples whose sequences had been downloaded from GenBank. In addition, a second unrooted phylogeny showing the relationship among only the new samples was calculated separately by the software without including sequences from GenBank.

In order to determine if nucleotide variants would result in translational differences, the amino acid sequence and corresponding codons for TRIM62 (Figure 2) were inspected. Using a

codon table, a mutation at base 408 and 491 of the TRIM62 gene were evaluated for an amino acid change.

Statistical analysis

The association of LPDV infection (yes or no) with TRIM62 exon 3 sequence variants was tested for statistical significance with a two-tailed, Fisher's Exact Test with alpha = 0.05 (https://www.graphpad.com/quickcalcs/contingency1/).

Results

Infection survey

Over the span of three years (2018, 2019, and 2020), a total of 44 wild turkey sampling kits were collected from volunteers. These birds were harvested during the spring hunting seasons in Mississippi, Tennessee, and Oklahoma. Out of the 44 samples, 22 tested positive for LPDV infection (50%). The primer pair designed by Allison et al. (2014) indicated 18 of these cases, and the primer pair recommended by Dr. Randal Renshaw indicated 4 cases. For more information on the 2018 and 2019 samples, see Hyche (2019). The 2020 samples were collected from 15 birds in West Tennessee from the following counties: Carroll, Chester, Gibson, and McNairy. Out of the 15 samples I collected, 3 tested positive for LPDV infection (20%) with one being from Carroll county and two from Chester County. The average weight of adult turkeys that were infected with LPDV (8.79 kg) was slightly lower than the average of uninfected adults (9.61 kg). Adults were more likely to be infected; 2 juveniles were sampled and neither showed infection. Additionally, turkeys living in crop field habitats were more likely to be infected than those living in any other habitat (Figure 4).

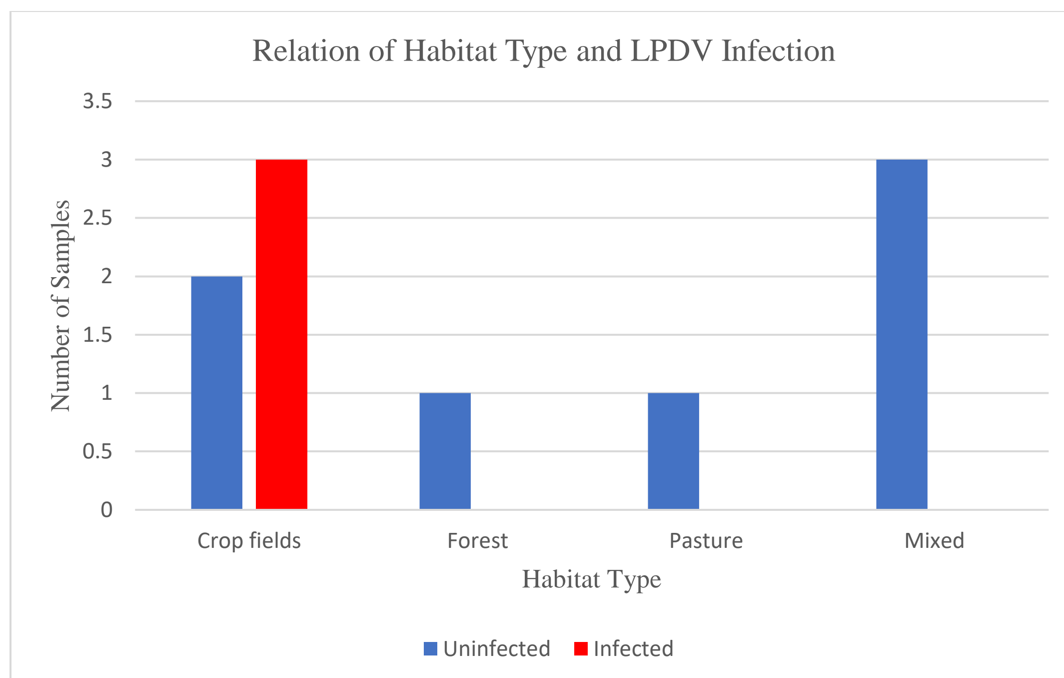
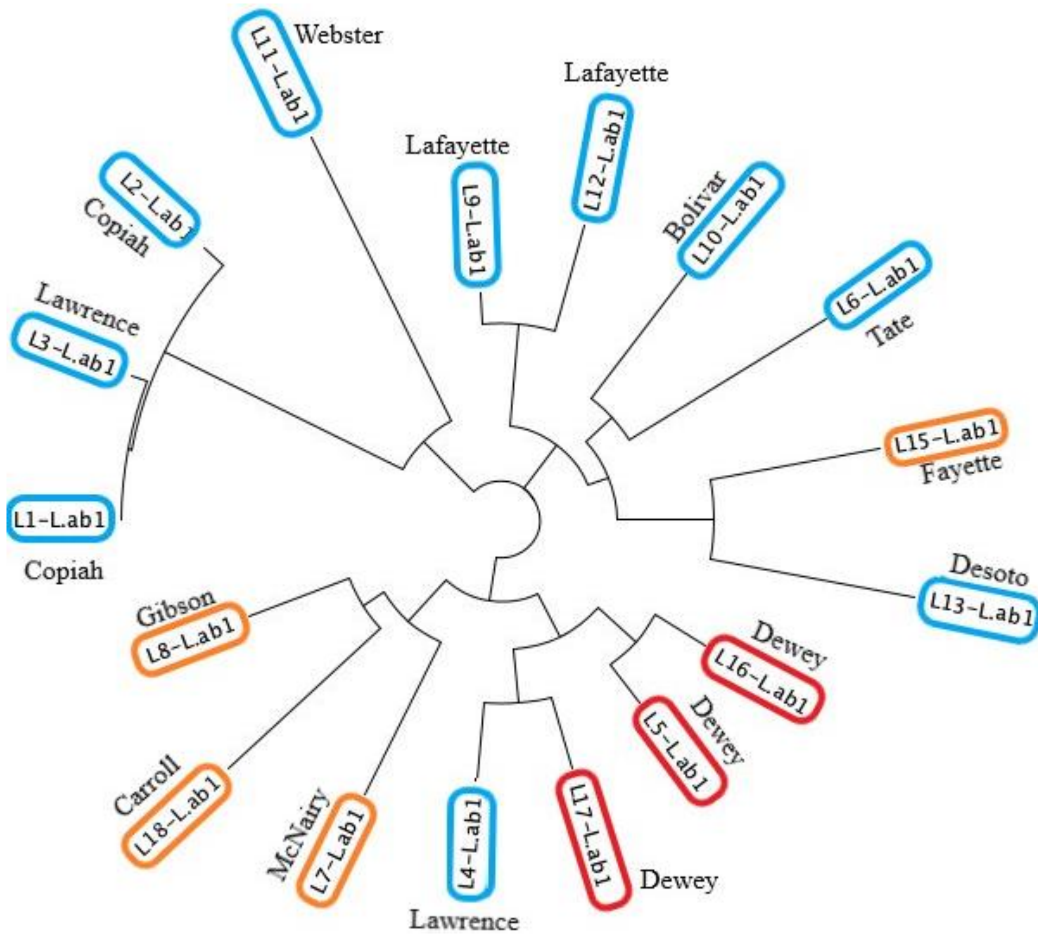


Figure 4: Hunters collected samples from a variety of habitat types, but the only samples that tested positive for LPDV came from crop fields. Sample size shown in Figure is only 10 because five hunters did not record habitat type.

LPDV sequences

Out of the 22 LPDV samples that were submitted, 21 were successfully sequenced. The region of the genome amplified by the second primer pair (from Dr. Renshaw) did not overlap with the first primer pair, and due to the small sample size, these sequences were not included in the phylogenetic analysis. Among the 17 LPDV sequences of the *gag* gene, the genetic distance of the virus samples was clustered by state except for two instances. One included a virus from Mississippi and a virus from Tennessee being more closely related to each other than any of the other viruses, and the other had a virus from Mississippi and a virus from Oklahoma being each other's nearest genetic samples (Figure 5). When examined at a broader geographic scale (Table 1), not all of my samples show a nearest viral relative from the same US state or even the same country. Unexpected similarity pairings include: a Mississippi LPDV sequence that associated

most closely with a GenBank sequence from New York, an Oklahoma sequence most similar to a GenBank sequence of a virus sample collected from Florida, and Tennessee sequences showing least genetic distance to GenBank sequences from Manitoba, Canada.



<u>Figure 5</u>: This phylogenetic tree shows the relationship between the LPDV samples collected in this study. Blue represents viruses from Mississippi, orange represents viruses from Tennessee, and red represents viruses from Oklahoma. Names of the counties where the bird was harvested is also included.

Table 1: This table shows the closest genetic relative of the collected LPDV samples from a phylogeny based on partial *gag* gene sequences, excluding the closest relatives that were collected in this study. MRA# is the Genbank accession number of the Genbank sequence that showed nearest phylogenetic distance to the sample collected in this study.

Sample	State, County	Year	Most Related (MRA#)	MRA Location	MRA Year
L10	MS, Bolivar	2019	KC801963	MS	2012
L1	MS, Copiah	2018	KC801974	MS	2012
L3	MS, Copiah	2018	KC801974	MS	2012
L13	MS, Desoto	2019	KC801972	MS	2012
L9	MS, Lafayette	2019	KC801959	NJ	2012
L12	MS, Lafayette	2019	KU211594	NY	2014
L2	MS, Lawrence	2018	KC801974	MS	2012
L4	MS, Lawrence	2018	KP299726	KS	2013
L6	MS, Tate	2019	KC801972	FL	2013
L11	MS, Webster	2019	KC801972	MS	2012
L5	OK, Dewey	2019	KP299712	FL	2013
L16	OK, Dewey	2019	KP299712	FL	2013
L17	OK, Dewey	2019	KP299726	KS	2013
L18	TN, Carroll	2020	MK548381	Manitoba, Canada	2018
L15	TN, Fayette	2019	KC801972	MS	2012
L8	TN, Gibson	2019	MK548381	Manitoba, Canada	2018
L7	TN, McNairy	2019	MK548381	Manitoba, Canada	2018

TRIM62 fragment sequences

Attempts to amplify additional exons and introns of the TRIM62 gene for sequencing were unsuccessful (Table 2) despite attempts to optimize PCR conditions. All but one out of the 44 amplicons of primer pair #6 were sequenced successfully. Analysis of the sequences showed that the consensus sequence matched the reference sequence (NCBI Reference Sequence: NC_015033.2), and there were two points of variation within the TRIM62 gene fragment. At base 408 of the gene, 10 samples contained a cytosine (C) instead of a thymine (T). The second point of variation was at base 491 of the gene, where two samples had a T instead of a C. One of these samples was infected and the other was not. Seven out of 10 base 408 variants were

infected compared to 15 of 33 consensus matches showing infection. This demonstrates that there was no significant relationship between variation in exon 3 and LPDV infection status (Fisher's Exact Test, P =0.2806). Base 408 of the TRIM62 gene is within the codon that codes for the 136th amino acid, glutamine, and base 491 is within the codon that codes for the 164th amino acid, leucine. The sequence variants at these positions (C instead of T at 408 and T instead of C at 491) would be silent mutations in that the codons would still result in the same amino acid being encoded as the consensus sequence.

<u>Table 2</u>: Outcomes of using the primer pairs for the TRIM62 gene that were suggested using the NCBI Primer Blast tool are shown. The base positions are in reference to NCBI Reference Sequence: NC_015033.2. Primer pair 6 produced amplicons that were suitable for sequencing.

Primer Pair	Forward and Reverse Primer Sequences (5'-3')	Base Position Start/Stop	Exon Coverage	Outcome
1	F: ATGATGGATATCTATGGGGCTG R: CGCTCCTACCTTGGTGTC	4332122/ 4332442	All of 2	Primer self-amplification
2	F: CCGACACCAAGGTAGGAG R: GAAGTCTTCGTAGGTCAGGTT	4332423/ 4332909	End of 2, all of 3, beginning of 4	Multiple Bands
3	F: CCCATACAATCCCCTTGTCTAT R: CTCGCTCCTACCTTGGTGT	4331825/ 4332444	All of 2	Multiple Bands
4	F: TACAATCCCCTTGTCTATCCC R: TCGCTCCTACCTTGGTGTC	4331829/ 4332443	All of 2	Multiple Bands
5	F: GATGGATATCTATGGGGCTGT R: CTCGCTCCTACCTTGGTGTC	4332124/ 4332444	All of 2	Multiple Bands
6	F: ACACCAAGGTAGGAGCGAG R: GAAGTCTTCGTAGGTCAGGTTG	4332426/ 4332909	End of 2, all of 3, beginning of 4	Single band of correct size

<u>Discussion</u>

LPDV infection percentage and geographic distribution

Through information gathered in previous studies, as well as this one, it has become clear that LPDV is widespread in wild turkey populations throughout North America. When my results are

combined with those of Hyche (2019), 50% of sampled turkeys from three different states were infected with LPDV. This outcome is consistent with results from previous LPDV studies (Table 3). It is interesting to see that four birds that tested negative for LPDV using the Allison et al. (2014) primer pair tested positive for LPDV using the Renshaw primer pair. This suggests that LPDV strains circulating in the US may have different lineages and could occur as co-infections in single birds. Unfortunately, I did not have the entire gene sequence of the Renshaw-primer-positive viruses in order to compare their sequences at the *gag* gene covered by the Allison et al. (2014) primers.

Table 3: Infection rates in wild turkey populations from southern states or in studies that included southern states are lowest and most similar. [a] Sample pool includes samples previously tested by Hyche (2019).

Author Citation	Total Infection Percentage	States Included
This Study[a]	50%	MS, TN, OK
Allison et al (2014)	44.6%	SC
Thomas et al. (2015)	47%	SC, WV, NY, VA, FL, LA, OK, NJ, MO, GA, NH, VT, KS, MA, ME, RI, NC
Alger et al. (2017)	55%	NY
Hyche (2019)	40%	MS
MacDonald et al. (2019)	65%	Ontario, Canada

My study shows that there is some geographic clustering of LPDV in the United States, but gene flow is not restricted. Geographically disconnected distributions of closely related LPDV samples may result from historical restoration efforts of translocating birds between states and from the US to Canada. The most striking example is the closest genetic relative to three out of the four samples that came from Tennessee is from Manitoba, Canada. As mentioned by MacDonald et al. (2019), wild turkeys have been reintroduced to Canada using birds translocated from various states, including Tennessee. For this reason, review of methods used in restoration

and management plans for ensuring that only healthy individuals are used in translocations is warranted.

TRIM62 gene variation

This study marks the first investigation of genetic variation of a TRIM gene among individual wild birds of any species. I hypothesized that genetic variation in the TRIM62 gene would be related to LPDV infection status. Base 408 variants still resulted in glutamine being encoded by the codon; therefore, this mutation would be considered "silent" in that the TRIM protein configuration would not change due to translation of a different amino acid. Thus, it would have been surprising if I had found a significant relationship between variation in the TRIM62 fragment studied and LPDV infection status. Findings of the turkey genome project showed that the TRIM62 gene in wild turkeys does not contain the SPRY domain that was found to be crucial in restricting ALV replication in chickens (Li et al. 2019). Perhaps, lacking this domain, wild turkey TRIM62 has no significant role in defending against retroviruses such as ALV and LPDV.

Conclusions and Future Research

Although the LPDV is found in one-half of wild turkeys sampled so far, it is still unclear whether LPDV is impacting wild turkey populations negatively. Previous researchers have shown that histological examination of healthy-looking birds reveals notable tissue disruption of crucial organ systems (Biggs et al. 1978; Allison et al. 2014; Thomas et al. 2015). It has also been shown that infected, adult males are underweight compared to the normal range (Hyche 2019). Results in this study also show that the average weight of infected birds is slightly less than uninfected birds, suggesting that LPDV infection may be related to poorer body conditions. What researchers have not investigated is whether infected birds have poorer reproduction or

shorter lifespans than uninfected birds. Of particular interest would be to elucidate when and how wild turkeys become infected. Young animals tend to be more susceptible to disease. The death of young turkey poults due to vertical or horizontal transmission from their mothers, however, would not be easily detected since dead chicks are quickly removed by scavengers in the wild.

Although lymphoproliferative disease was first described in wild turkeys in European nations, the exact origin of LPDV is still unknown (Bigs et al. 2019). It is interesting to see that most LPDV samples collected from the United States belong to a North American clade. Only a minute percentage have been grouped separately with the original Israeli prototype strain suggesting limited transmission of this lineage (Thomas et al. 2015), perhaps only where wild turkeys have been exposed to domestic poultry. Alternatively, mutations of the original Israeli strain may have allowed for the emergence and spread of the North American viral clade. Probably complete genome analysis of LPDV samples from throughout the USA and abroad will be needed to understand the origins and colonization paths of this virus.

New viruses continue to be discovered, even in rather well studied species of birds. Such viruses may be widespread but only cause host mortality under unique circumstances. For example, Goldberg et al. (2019) conducted a study investigating the cause of unexplained high rates of bald eagle (*Haliaeetus leucocephalus*) mortality in a certain region of Wisconsin. Wisconsin River Eagle Syndrome (WRES) is associated with severe neurological effects in eagles surrounding the lower Wisconsin River and eventually leads to death. This region of the Wisconsin River does not freeze during the winter allowing eagles easier access to prey. The large gatherings of birds during this time period coincides with occurrences of WRES. The authors found that a novel virus, bald eagle hepacivirus (BeHV), occurs in 31.9% of eagles

sampled throughout the USA but appears to be associated with WRES primarily at the lower Wisconsin River location. As with wild turkeys and LPDV, more research is needed to determine the extent of the negative effect that this novel virus is having on bald eagle populations (Goldberg et al. 2019).

My study of TRIM gene sequence variation is the first of its kind for a wild bird species, but merely scratches the surface of a largely unexplored area of the avian genome. A comprehensive investigation of TRIM genes in wild turkeys may give insight into other genes or gene regions that affect individual susceptibility to certain viruses. With wild turkey populations declining, an additional focus on how landscape features and genetic variation in the host interact with transmission dynamics of LPDV and other pathogens may be critical to the conservation of sustainably hunted populations of this species.

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