Molecular Surveillance for Lymphoproliferative Disease Virus in Hunter-Killed Wild Turkeys (M*eleagris gallopavo*) from the Eastern United States

By

Jesse Michael Thomas

(Under the Direction of Michael J. Yabsley)

ABSTRACT

Three avian retroviruses can cause lymphoid tumors in galliforms, including avian leukosis virus, reticuloendotheliosis virus, and lymphoproliferative disease virus (LPDV). Historically, LPDV was considered a poultry pathogen of minor importance. However, in 2009, LPDV was first detected in three wild turkeys (*Meleagris gallopavo)* with evidence of lymphotumoral disease. Currently, little is known about the epidemiology, natural history, transmission, or pathobiology of LPDV. To begin to address these gaps, we surveyed asymptomatic hunter-killed wild turkeys for evidence of LPDV proviral DNA. Overall, 1,164 wild turkeys were tested from 417 counties throughout 17 states, detecting 564 positives for a prevalence estimate of 47%. Paired liver, spleen, and bone marrow samples were also screened (n=35) to determine the best tissue to target for future diagnostic and surveillance efforts. Of these tissues, bone marrow was the most efficient. These results suggest subclinical LPDV infection is common in wild turkeys throughout the Eastern United States.

INDEX WORDS: Lymphoproliferative Disease Virus, LPDV, polymerase chain reaction, proviral DNA, wild turkey

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CHAPTER 1

INTRODUCTION

Avian retroviruses can induce a variety neoplastic diseases in wild and domestic galliforms. Three avian retroviruses are recognized pathogens of domestic poultry: avian leukosis/sarcoma virus (ALSV), reticuloendotheliosis virus (REV), and lymphoproliferative disease virus (LPDV). Experimentally, ALSV and REV can infect a wide range of birds, but ALSV and REV typically only cause clinical disease in chickens and chickens and turkeys, respectively. Prior to 2009, LPDV had only been reported in domestic turkeys.

Lymphoproliferative disease virus is an uncommon viral pathogen of domestic turkeys that causes lymphoid neoplasia characterized by proliferation of pleomorphic lymphoid cells in multiple organs (Biggs et al 1978a). First described in the United Kingdom in 1972, sporadic outbreaks have also been reported in domestic turkeys in other European countries and Israel (Gazit and Yaniv 1999). As LPDV sequences are not present uninfected turkey cells, the virus is not considered an endogenous retrovirus of domestic turkeys (Payne 1998). The prevalence and epidemiology of LPDV is not well documented, likely due to rarity of lymphotumoral disease in domestic poultry and limited diagnostic tools available for LPDV detection. In regards to the latter, it is not known how to culture LPDV *in vitro*; therefore, detection currently relies on on histopathology to identify lymphoid tumors and molecular assays, such as polymerase chain reaction (PCR), to detect the virus.

Historically, LPDV has not been reported from wild or domestic galliforms in North America; however, the virus was recently detected in multiple wild turkeys (*Meleagris*

gallopavo) from throughout the Eastern United States during 2009-2012 (Allison et al 2013). Currently, there are many questions relating to LPDV in wild turkeys. To start to address some of these unknowns, the goals of this study were to determine the prevalence and geographic distribution of LPDV infection in asymptomatic wild turkeys throughout the Eastern United States and identify the most appropriate tissue for molecular testing. Toward these goals, our specific objectives included:

Objective 1: Determine the prevalence and geographic distribution of LPDV in hunter-killed wild turkeys throughout the Eastern U.S.

Hypothesis: Based upon previous testing of wild turkey diagnostic cases, it is hypothesized that LPDV infection will be widespread in asymptomatic hunter-killed wild turkeys and that the statewide prevalences will be high (>40%).

Objective 2: Determine the most appropriate tissue for molecular detection of LPDV in hunter-killed wild turkeys and wild turkey diagnostic cases.

Hypothesis: Based on existing data on LPDV viral tropism (lymphoid cells) and previous results from wild turkey diagnostic cases, it is hypothesized that bone marrow will be the most appropriate tissue for molecular detection of LPDV.

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CHAPTER 2

LYMPHOPROLIFERATIVE DISEASE VIRUS—LITERATURE REVIEW

Description

LPDV is an oncovirus in the family *Retroviridae* and subfamily *Oncovirinae*. Both circumstantial and experimental evidence strongly indicates that it is a type C oncovirus (Biggs 1997). Budding of type C virus particles has been demonstrated from the surface of tumor cells in domestic turkeys with lymphoproliferative disease and type C particles have been isolated from the blood of experimentally LPDV-infected turkeys (Perk et al 1978, Yaniv et al 1979). The genome of LPDV (7.1 Kb) has been fully sequenced and, similar to other retroviruses, contains a primer binding site (PBS),*gag*, *pro*, *pol*, and *env* genes, and several open reading frames (ORF) with unknown functions (Figure 1).

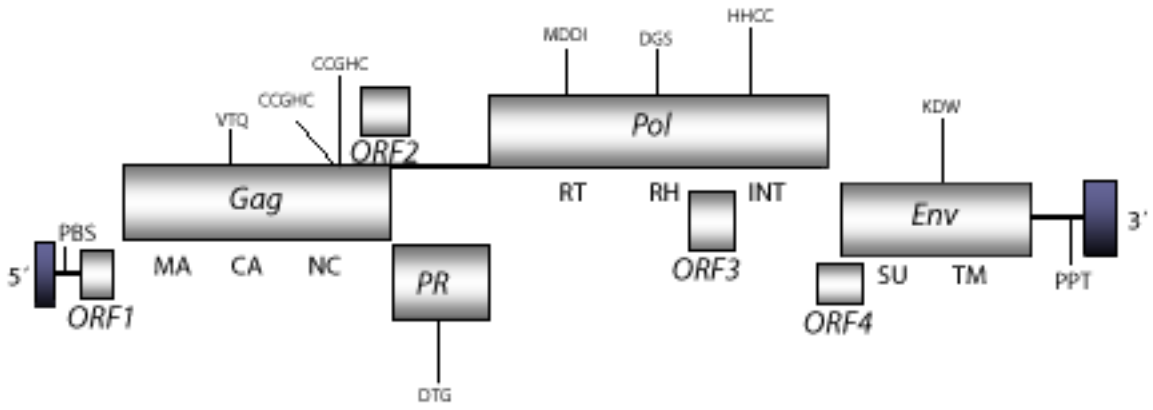

Figure 2.1.Genomic organization of proviral LPDV (Biggs 1997).

Genetic analyses indicate that LPDV is genetically distinct, but most similar to alpharetroviruses, avian leukosis/sarcoma virus (ALSV) and reticuloendotheliosis viruses (REV)

(Biggs 1997; Yaniv et al 1979; Gak et al 1989). The topologies of the trees based on the *pol* gene, which is the most conserved retroviral structural gene, from Chajut et al 1992 agree, in general, with those derived from other studies (Figure 2).

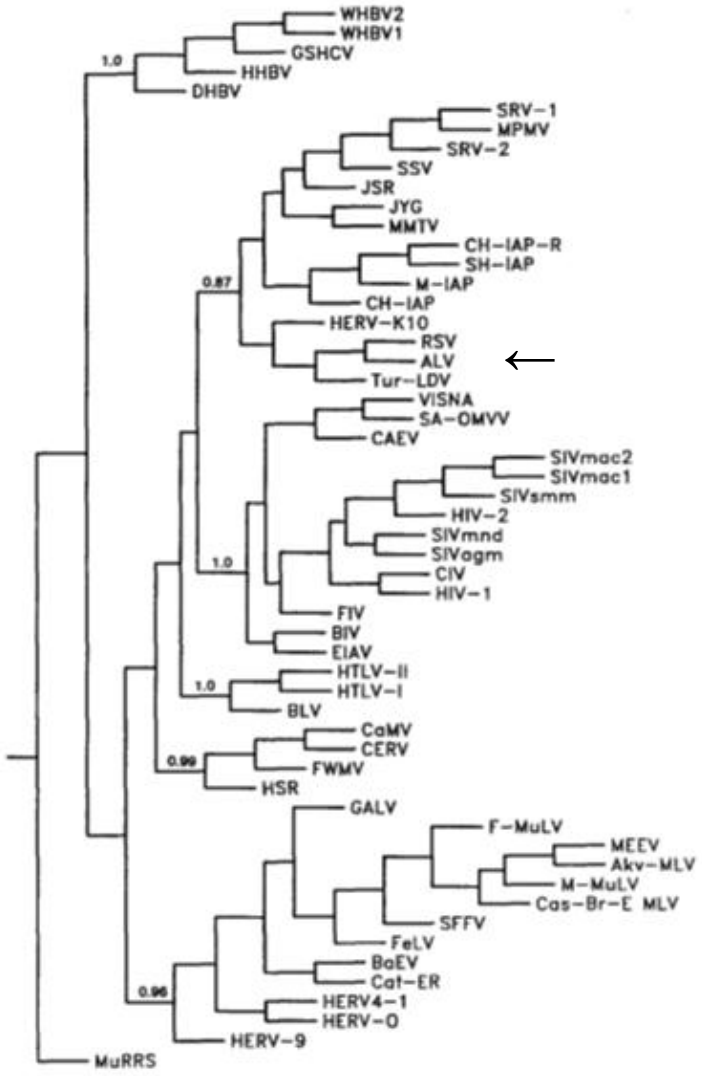

Figure 2.2. Rooted evolutionary tree of 55 retroelements based on the nucleotide sequences of the *pol* gene, constructed with the maximum-parsimony method. Rous sarcoma virus (RSV), avian leukosis virus (ALV), lymphoproliferative disease virus (LDV) (Llorens 2011).

To date, research on LPDV has been limited because of the sporadic occurrence of disease outbreaks in domestic turkeys and limited diagnostic tools for detecting infection, which currently relies largely on polymerase chain reaction to detect proviral DNA. To date, attempts to propagate the virus *in vitro* have been unsuccessful. Various culture systems for LPDV have been tested, including embryo fibroblasts of turkeys, ducks, quail, chickens, and kidney cells of chicks and turkeys (McDougall et al 1978). In house serologic assays have been described, but are not widely available (Patel and Shilleto 1987).

Natural and Experimental Infections in Domestic Turkeys

Natural infections of domestic turkeys with LPDV were first reported in the United Kingdom during outbreaks of lymphotumoral disease, followed by Austria, Israel, and other European countries (Gazit and Yaniv 1999). Viral infection induces lymphoid tumors involving multiple organs, but most commonly the spleen, thymus and liver (Gazit and Yaniv 1999). A series of outbreaks in 1972, which occurred in several flocks owned by one turkey-growing organization in the United Kingdom, had clinical presentations and gross and microscopic lesions consistent with LPDV (Biggs et al 1978a). Few non-specific clinical signs were observed (i.e., ruffled feathers, anorexia, and reluctance to move), but often birds were found dead with no premonitory signs(Biggs et al 1978a). Gross lesions included hepatomegaly, splenomegaly, marbled and pale appearance of spleen, numerous miliary, greyish white foci in the liver, as well as pale tan tumors variably present in the kidneys, gonads, intestinal wall, pancreas, lungs, and myocardium (Biggs et al 1978). Microscopically, these neoplastic lesions consist of large numbers of pleomorphic lymphoid cells that completely efface the normal parenchuma. The cellular infiltrate consists of lymphocytes, lymphoblasts, reticulum cells and plasma cells. (Biggs et al 1978).

Several experimental studies have investigated the susceptibility of domestic turkeys to infection with LPDV (McDougall et al 1978, Gazit et al 1982, Gazit et al 1983a, Zimber et al 1983, Ianconescu et al 1981, Zimber et al 1984, Patel and Shilleto 1987). In one study, 40 four-week-old poults were inoculated with spleen homogenate collected from domestic turkeys during a natural LPDV outbreak with lymphotumoral disease. A second group of 40, age-matched poults served as contact controls. After 9 weeks, 30% and 18% of the inoculated and contact birds, respectively, exhibited clinical disease and microscopic lesions consistent with natural LPDV infection (i.e. lymphoid neoplasia; McDougall et. al 1978). In order to rule out other causes, all birds were tested for antibodies to other avian tumor viruses, including ALV, REV, Marek's disease virus, and herpesvirus of turkeys. These data indicated that the virus could be transmitted by inoculation of infected tissue and that the virus could spread horizontally between birds in direct contact (Gazit and Yaniv 1999). Disease in naturally-exposed domestic turkeys typically occurs between 7 and 18 weeks of age. Currently, the only method of control is depopulation of infected turkey flocks (Payne 1998). Experimental trials have also shown that different domestic turkey breeds vary in their susceptibility to LPDV infection as prevalence of infection ranged from 4-36% in four different turkey strains (McDougall et al 1978). In one study conducted by Patel and Shilleto 1987, no significant differences in the circulating concentration of LPDV-infected leukocytes and cell-free virus was detected in samples collected at four weeks or 16 months after virus inoculation, which suggests that turkeys can develop long-term asymptomatic viraemia.

Experimental inoculation of domestic ducks and geese with LPDV did not result in infections, and although chickens did become infected, gross LPDV-like lesions, such as enlarged spleen and/or liver and congested thymus, were rare (Ianconescu 1983; Gazit et al

1983b). However, microscopic lesions consistent with LPDV infection were seen in the spleen, liver, pancreas, and thymus in a number of infected chickens (Gazit et al 1983b).

Other viruses that infect turkeys and cause similar lesions

Reticuloendotheliosis virus (REV)

Due to the fact that REV infection has been reported in wild upland game birds in the United States and sporadically produce similar lesions (lymphoid neoplasia), a general review of this virus is provided. REV belongs to the genus *Mammalian C-type* within the family *Retroviridae* and subfamily *Orthoretrovirinae*. Based upon host immunological responses, ultrastructure, morphology, and nucleic acid sequences, REV is distinct from ALV (Coffin 1996). REV can be transmitted from infected birds to naïve birds by both vertical and horizontal transmission, but the primary route of transmission in commercial operations is unclear (Witter 1991).

Natural REV infections have been reported in domestic and wild turkeys, ducks, geese, chickens, and Japanese quail (*Coturnix japonica*); however, domestic turkeys have been most frequently observed with overt disease (Witter 1984). Lesions associated with REV infection generally vary from enlargements of the spleen, liver, heart, thymus, or bursa to nodular lymphomas in visceral organs with or without necrosis (Witter and Fadly 2003). REV has been isolated from the spleen and liver of infected wild turkeys using virus isolation (Hayes et al 1992; Ley et al 1989; Peterson et al 2002) and proviral DNA has been detected by PCR assay from apparently asymptomatic wild turkeys, including 2/70 sampled in the Edwards Plateau of Texas in 2002 (Peterson et al 2002).

REV, along with other retroviruses, can be suspected based on gross and microscopic lesions, but infection must be confirmed through ancillary diagnostics including virus isolation,

detection of REV antigen or nucleic acid, or presence of viral antibodies in affected birds (Drew 2007). Similarly, avian pox can be diagnosed by gross and microscopic lesions followed by confirmation by viral culture (Riper and Forrester 2007). Unfortunately, LPDV cannot currently be cultured *in vitro*, which is a major limitation when performing diagnostics. Due to this limitation, diagnosis must rely on histopathology and molecular assays, such as polymerase chain reaction.

Avian fowl pox virus (AFPV)

Avian pox is caused by viruses of the genus *Avipoxvirus* in the family Poxviridae. Lesions most commonly associated with avian pox are located on unfeathered parts of the body, including the legs, feet, eyelids, base of the beak, and the comb and wattles of gallinaceous birds. Microscopically, lesions associated with avian pox are localized proliferations of epithelial cells, and affected cells become hyperplastic and hypertrophic due to the increased rate of multiplication of the basal germinal layer of cells within the epithelium (Riper and Forrester 2007). In addition, large cytoplasmic viral inclusion bodies can be found in the cytoplasm of epithelial cells. Therefore, while LPDV and avian pox may look similarly grossly, they are very different when looked at microscopically.

Natural Infection in free-ranging Eastern wild turkeys

In 2009, LPDV was first identified in the United States in three adult Eastern wild turkeys with evidence of lymphoid neoplasia (Allison et al 2013). Histologically, these wild turkeys had lymphoid tumors in multiple organs, which microscopically were consistent with LPDV-induced neoplasia in domestic turkeys .Tissue samples from all three cases were negative for REV and other endogenous oncogenic viruses by virus isolation and PCR. However spleen and liver from all three cases were positive for LPDV proviral DNA by PCR, which were

confirmed by sequencing a portion of the *gag* polyprotein. Since these cases, subsequent testing of wild turkeys submitted for diagnostic examination identified an additional 38wild turkeys from 19 states that were positive for LPDV proviral DNA by PCR. Only a small number (n=6) of these 38 LPDV-positive turkeys had evidence of neoplasia and most had other causes of disease, including avian pox, bacterial infection, trauma, toxicosis, and parasitism (Allison et al 2013).

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CHAPTER 3

Molecular Surveillance for Lymphoproliferative Disease Virus in Hunter-Killed Wild Turkeys (M*eleagris gallopavo*) from the Eastern United States

Thomas JM, Phillips JE, Bunting EM, Allison AB, Yabsley MJ, Brown, JD. Molecular Surveillance for Lymphoproliferative Disease Virus in Hunter Killed Wild Turkeys (*Meleagris gallopavo*) from the Eastern United States. Formatted for submission to Journal of Wildlife Diseases.

ABSTRACT

Avian retroviruses can induce a variety of neoplastic diseases in wild and domestic galliforms and are a source of significant economic losses to the poultry industry worldwide. Lymphoproliferative disease virus (LPDV) of domestic turkeys is an uncommon retroviral pathogen that induces lymphoid tumors characterized by the proliferation of pleomorphic lymphoid cells in multiple organs. Historically, LPDV has only been recognized in domestic turkeys and has been geographically restricted to Europe and Israel. Following the recent identification of LPDV in wild turkeys (*Meleagris gallopavo*) in the United States during 2009, we surveyed 1,164 apparently asymptomatic hunter-killed wild turkeys from 17 states located in the Eastern U.S. for the presence of LPDV proviral DNA by polymerase chain reaction (PCR). A total of 564 (47%) turkeys were PCR positive for LPDV. Positive wild turkeys were identified in all 17 states, with state-wide prevalences ranging from 26% to 83%. Additionally, in order to determine the best tissue for molecular detection of LPDV in wild turkeys, multiple tissues (liver, spleen, and bone marrow) were tested from 35 LPDV-positive wild turkeys, including from 15 asymptomatic hunter-killed birds and 20 turkeys submitted to a diagnostic laboratory for disease investigations. Bone marrow was positive in all LPDV-positive hunter-killed turkeys and diagnostic cases. Spleen and liver were only positive in a subset of the LPDV-positive turkeys; differences between bone marrow and liver or spleen were significant for hunter-killed turkeys but not for diagnostic cases. Based on these diagnostic results, LPDV infection appears to be widespread in wild turkeys throughout the Eastern United States, even in the absence of overt disease (i.e. neoplasia).

Key words: Lymphoproliferative disease virus, LPDV, polymerase chain reaction, proviral DNA, wild turkey

INTRODUCTION

Neoplastic diseases of domestic poultry can be non-infectious (i.e. spontaneous) or induced by viruses. Non-infectious neoplasms are sporadic, typically occur in older birds, often are of epithelial cell-origin, and are of minimal economic significance to the poultry industry (Fadly 2008). Viral-induced neoplasms in domestic poultry are most commonly caused by three viruses: 1) Gallidherpesvirus 2 (i.e. Marek's disease virus (MDV)), 2) avian leukosis virus (ALV), and 3) reticuloendotheliosis virus (REV). Marek's disease virus is an alphaherpesvirus (Genus *Mardivirus*) that produces lymphoproliferative disease in domestic chickens and, occasionally, domestic turkeys, quail, and pheasants (Calnek and Witter 1997). The virus is highly contagious and can be transmitted horizontally through the inhalation of MDV-contaminated dust in poultry houses (Payne 2000). Marek's disease virus is ubiquitous in domestic chicken populations throughout the world and is a significant source of economic losses due to mortality, condemnation, decreased production, and/or costs associated with control (Payne and Venugopal 2000). Since the introduction of vaccination of commercial poultry in the early 1970's, mortality associated with MDV has dropped significantly; however, sporadic outbreaks still occur as new virulent viral strains emerge for which the vaccines are not fully protective (Payne and Venugopal 2000). Avian leukosis virus is an alpharetrovirus (Genus *Alpharetrovirus*) that produces a wide-variety of hemopoietic and connective tissue tumors in domestic chickens, but the most common neoplasms are lymphoid leukosis and myeloid leukosis (Fadly and Nair 2008). Transmission of ALV can occur either vertically (from infected hen to offspring via the egg) (Cottral et al 1954) or horizontally through direct or indirect exposure (Rubin et al 1961, 1962; Payne 2003). Similar to MDV, ALV is ubiquitous in domestic chicken populations worldwide; however, the incidence of neoplasms or other overt signs of disease in infected flocks is typically low (1-2%) (Fadly and Nair 2008). Despite the relatively low

incidence of morbidity and mortality, ALV is still considered to have significant economic impacts on poultry due to losses associated with mortality, condemnation, and reduced production (Fadly and Nair 2008). Reticuloendotheliosis virus is a gammaretrovirus (Genus *Gammaretrovirus*) that produces several neoplastic and non-neoplastic disease syndromes in birds, including runting syndrome, chronic lymphoid neoplasms, and acute reticulum cell neoplasia (Witter and Fadly 2008). As opposed to ALV and MDV, which are predominately pathogens of chickens, REV can cause disease in a variety of wild and domestic avian species, including domestic and wild turkeys (*Meleagris gallopavo*), chickens, ducks, geese, pheasants, quail, peafowl, and prairie chickens (*Tympanuchus* spp.) (Witter 1984; Chen et al 1987; Witter 1991; Drew et al 1998). Similar to ALV, REV can be transmitted vertically or horizontally through direct or indirect routes (Drew 2007). Although REV infection is widespread and common in domestic poultry in several countries, overt disease, including tumors or runting syndrome, is uncommon and most infections are presumably silent (Payne 1998). Consequently, the economic importance of REV for the poultry industry is considered to be relatively minor.

A rare neoplastic syndrome of domestic turkeys called lymphoproliferative disease is caused by a distinct retrovirus, lymphoproliferative disease virus (LPDV). Outbreaks of LPDV in domestic turkeys have been extremely rare and only reported in a few European countries and Israel (Gazit and Yaniv, 1999; Fadly 2003). The characteristic lesion of lymphoproliferative disease is pleomorphic lymphoid cells, including lymphocytes, lymphoblasts, reticulum cells, and plasma cells, infiltrating multiple visceral organs and tissues. Mortality during LPDV outbreaks has typically been low and young poults, between 7 and 18 weeks of age, have been the most severely affected (Gazit and Yaniv 1999; Biggs 1997). The natural routes of LPDV transmission in domestic turkeys are not currently known. Experimentally, LPDV can be

transmitted horizontally between poults in direct contact, but the primary transmission route has not been identified. Additionally, it is unknown if LPDV can be transmitted vertically. To date, LPDV has not been cultured *in vitro*, which has significantly limited surveillance, diagnostics, and research efforts on this virus.

In domestic poultry, retroviral-induced neoplasms are inherently challenging to study as different viruses can induce similar tumors and lesions, individual virus can produce a variety of neoplastic syndromes, and infection in domestic poultry is often widespread in the absence of overt disease. These challenges are compounded in wild birds by a lack of surveillance data on incidence or epidemiology of retroviruses in wild bird populations. In general, neoplasia in wild birds is rare (Jennings 1968; Siegfried 1983; Drew 2007). Lymphoproliferative neoplasms have sporadically been reported in in a variety of wild upland game birds, including wild turkeys, ring-necked pheasants (*Phasianuscolchicus*), and prairie chickens (*Tympanuchuscupido*) (Ley et al 1989; Hayes et al. 1992; Busch and Williams, 1970; Colwell et al., 1973; Grant et al., 1975; Davidson et. al 1985; Hanson and Howell 1979; Dren et al., 1983; Drew et al., 1998; Davidson 2006). No causative virus was identified from most of these cases; however, REV has reportedly been detected from a wild turkey, ring-necked pheasant, Japanese quail (*Coturnix japonica*), and Attwater's and greater prairie chickens with lymphoma (Hanson and Howell 1979; Dren et al 1983; Drew et al 1998; Witter et al 1991).

Historically, LPDV had not been reported from wild birds or domestic poultry in North America. However, in 2009 LPDV was identified in an adult wild turkey from Arkansas with lymphoid tumors in multiple organs (Allison et al, 2013). Following this initial detection, testing of wild turkeys carcasses submitted to diagnostic laboratories for disease investigations identified LPDV infection in 17 states throughout the Eastern United States (U.S.) and as far

west as Colorado (Allison et al, 2013). Only six of these LPDV-positive wild turkeys had microscopic evidence of lymphoproliferative disease or neoplasia, and the majority had other unrelated causes of morbidity/mortality, including avian pox, bacterial infections, trauma, parasitism, or toxicosis. Additionally, in this study, a high prevalence (53%; 39/74) of LPDV was detected in liver samples collected from hunter-killed turkeys from South Carolina, providing preliminary evidence that infection may be common in wild turkeys without evidence of overt disease.

The goal of this research was to expand on the initial study by Allison et al. (2013) and determine the prevalence and distribution of LPDV infection in apparently asymptomatic wild turkeys throughout the Eastern U.S. Toward this goal, we tested tissues from 1,164 hunter-killed wild turkeys from 17 states for LPDV proviral DNA. Additionally, in an effort to facilitate future surveillance efforts, we tested multiple tissues (liver, spleen, and bone marrow) from a subset of wild turkeys to determine the most appropriate diagnostic sample from turkeys without evidence of lymphoproliferative disease.

METHODS

Sampling Methods

During the spring 2011, fall 2012, and spring 2013 wild turkey hunting seasons, samples were collected from hunter-killed wild turkeys in 417 counties from 17 states (Figure 1). New Jersey and Missouri were the only two states where samples were collected from two different hunting seasons (Fall 2012 and Spring 2013). The majority of the samples collected in this studywere derived from the Eastern wild turkey subspecies (*M. g. silvestris*), however, samples from the Osceola (*M. g. osceola*) and Rio Grande (*M. g. intermedia*) subspecies or hybrids with Eastern wild turkeys may have been included based on geographic origin of some samples

(subspecific identification was based on morphology and without confirmatory genetic testing). Tissue samples were obtained by one of two methods: 1) collection by state biologists at hunter check stations, or 2) collection by hunters who mailed the sample into their state agencies. Based on the known viral tropism (Biggs 1997), previous diagnostic experience with LPDV in wild turkeys (Allison et al., 2013) and the ease of organ identification, liver, spleen, and bone marrow were selected as the target tissues for this study. At least one of the three tissues was collected from each hunter-killed turkey; however, the specific tissue collected varied between states based on time and training of personnel, available resources, and existence of ongoing sampling efforts by the individual state agencies. Liver and spleen samples were placed into separate whirl-pak bags (Twirl'EmEcolo, Canada) and frozen at -20° C until testing. For bone marrow, the tibiotarsus was collected into individual ziploc bags and frozen at -20° C until testing. The harvest date and location (county, state) were recorded for all samples, and the age and gender of the turkey were recorded if they were determined. Gender and age determinations were based on a combination of morphological characteristics, including plumage, presence/absence of spurs, and/or tarsometatarsus measurements (Wakeling et al. 1997). For comparison, previously published data, using the same methodology, on prevalence of LPDV proviral DNA in hunter-killed wild turkey liver samples collected during spring 2011 in South Carolina were included in this analysis (Allison et al., 2013).

To evaluate the most appropriate tissue for LPDV testing in wild turkeys, paired samples of liver, spleen, and bone marrow were collected from 35 LPDV-positive wild turkeys, including 15 hunter-killed wild turkeys and 20 wild turkeys diagnostic cases submitted to the Southeastern Cooperative Wildlife Disease Study (SCWDS), College of Veterinary Medicine, the University of Georgia (Athens, Georgia, USA) for disease investigation. Birds were designated as positive

if one of the three tissues were positive for LPDV. All of the wild turkey diagnostic cases were found moribund or dead, but none had gross or microscopic evidence of lymphoproliferative disease.

Testing Methods

To reduce the chance of contamination during sample collection, all instruments utilized were first disinfected using Microban Germicidal Cleaner (Coraoplis, PA, USA) followed by bead sterilization at 240° C for 5 minutes. Bone marrow was extracted by fracturing the tibiotarsus with bone rongeurs and removing a sample of marrow with sterilized forceps. Liver and spleen samples were collected using sterile scalpel blades and forceps.

DNA was extracted from liver, spleen, and bone marrow samples using the Qiagen DNeasy Blood and Tissue Kit (Germantown, Maryland, USA). Extracted samples were tested for LPDV proviral DNA using published protocols and primers targeting a portion of the *gag* polyprotein (partial p31/partial CA; 431 nt) (Allison et. al, 2013). Stringent protocols and controls were utilized in all PCR assays to prevent and detect contamination. DNA extraction, amplification, and product analysis were performed in separate dedicated laboratory areas. A negative water control and known positive control was included in each set of PCR reactions. PCR products were visualized with gel electrophoresis (1.5% agarose gels), stained with ethidium bromide and visualized under ultraviolet light.

A subset of LPDV-positive samples (n= 41) were confirmed by sequencing the PCR product. Sequencing was conducted by the Georgia Genomics Facility, Athens, Georgia. Sequences were then put into BLAST and confirmed identity based on similarity to published sequences in GenBank (Allison et al 2013).

Data analysis

Results from each state were subcategorized by tissue and season of collection and analyzed by placing a 95% confidence interval around each prevalence estimate. Fisher's exact test was used to compare differences among age and gender classes, different tissue types for the paired sample testing, and geographic regions.

RESULTS

A total of 1,164 wild turkey samples from 417 counties in 17 states were tested for LPDV proviral DNA, of which 564 (47%) were positive (Table 1; Figure 1). Of the 41 LPDV positive samples that were sequenced, 39 (95%) were confirmed to be LPDV based on high similarity to existing sequences in GenBank (Allison et al., 2013). Positive wild turkeys were identified in all 17 states (Figure 1). Seasonal prevalence estimates for New Jersey and Missouri were as follows: New Jersey (Fall2012: 14/36 (39%); Spring 2013: 8/12 (67%); Missouri (Fall 2012: 14/39 (36%); Spring 2013: 8/35 (23%). No significant differences were noted between sampling seasons within each state ($p>0.05$).Overall state-wide prevalence estimates ranged from 26% in Oklahoma to 83% in New Hampshire (Table 1); however, there was extensive overlap of confidence intervals for the majority of the states. However, when analyzed by geographic region, overall prevalences in Northeastern states were significantly higher than the Mid-Atlantic and Southeast states, and the prevalence in the Central states was significantly lower than the other three regions (Table 2). When only states with bone marrow samples (n=653) were analyzed, similar differences by region were noted (Table 2). Overall, adult turkeys had a significantly higher prevalence than juveniles ($p=0.0001$) but there was no significant difference in prevalence between male and female turkeys ($p=0.81$; Figure 2). Due to the larger sample size in New York, regions 1-9 (established by the New York State Department of Environmental

Conservations) were analyzed for differences in prevalence rates. Region 2 was excluded from the analysis because there were no samples collected from that region. No significant differences were noted between regions ($p>0.05$).

The ability to detect LPDV proviral DNA in paired bone marrow, liver, and spleen samples varied between wild turkeys that were hunter-killed and those that were diagnostic cases (Table 3). There was no significant difference in detection between the tissue samples collected from diagnostic cases ($p > 0.10$), whereas in hunter-killed turkeys, the detection of LPDV was significantly higher in bone marrow compared to spleen ($p=0.04$) or liver ($p=0.01$).

DISCUSSION

Currently, data on the incidence, epidemiology, and significance of avian retroviruses in wild upland game birds is sparse and the majority of studies have focused on clinically-ill birds or laboratory-based challenge studies. Historically, retroviruses have rarely been identified in wild turkeys; however, during 2009 to 2012, LPDV was reported in multiple wild turkeys throughout the Eastern US (Allison et al., 2013). This current study expands on these initial detections through a large multi-state molecular survey for LPDV proviral DNA in hunter-killed wild turkeys. Our data suggest that LPDV in wild turkeys appears to follow the general paradigm of avian retroviruses in poultry, in which viral infection is common and widespread without clinical evidence of disease.

Consistent with the findings of Allison et al. (2013), LPDV infection was geographically widespread, with proviral DNA detected in every sampled state. Although there was variation between states, prevalence estimates were generally high. Combined with the previous report by Allison et al. (2013), the known distribution of LPDV in wild turkeys extends from Maine to Florida and as far west as Colorado, with LPDV detections reported in 24 states. While this distribution is consistent with the historic range of Eastern wild turkeys, it is largely a function of

where wild turkeys have been tested. Future LPDV surveillance efforts in wild turkeys outside of this range are warranted, and should include translocated Eastern wild turkeys and other subspecies. Regionally, the states in the Northeast had the highest overall prevalence of LPDV, followed by the Mid-Atlantic and Southern states, and finally the Central region. The reasons for these apparent regional differences in LPDV prevalence are not known. Additional studies are needed to not only confirm these results, but also further understand these spatial differences by investigating variables relating to sampling (collection, handling, and season), wild turkey biology (behavior, population densities), environment (habitat), and epidemiologic patterns of LPDV infection in wild turkeys.

Experimental and field studies on LPDV in domestic poultry have identified various host characteristics that can influence the susceptibility to infection or lymphoproliferative disease, including sex, age, breed, and species. In domestic turkeys, there has been some evidence to suggest males may be more susceptible to lymphoproliferative disease than females (Biggs 1997). Consistent with the results of Allison et al. (2013), there were no apparent differences in LPDV prevalence between male or female wild turkeys in this study. A strong effect of age on LPDV infection and lymphoproliferative disease has been observed in domestic turkeys. During outbreaks, the most severely affected age groups are young poults between 7 and 18 weeks of age, where cumulative flock mortality has reportedly reached 15-25% (Gazit and Yaniv, 1999). Lymphoproliferative disease in adult turkeys, when present, is less common with only sporadic mortality reported (Biggs 1997; Biggs et al 1978). Experimentally, poults inoculated with LPDV at 1-day-old had a lower incidence of lymphoproliferative disease than those challenged at 1-month-old (McDougall et al 1978). To date, it is unknown whether there are similar age-related impacts on LPDV infection in wild turkeys, as all of the work to date has been done on birds that

are 6 months or older, and younger poults rarely are submitted to diagnostic laboratories for disease investigations and are not hunted. However, the results of this current study did indicate AHY wild turkeys had a significantly higher prevalence of LPDV infection than HY birds. Additional surveillance and research on LPDV in young poults is needed in order to identify whether the virus is transmitted vertically and to better understand the population significance in wild turkeys by determining the incidence of lymphoproliferative disease in the most susceptible age class.

In domestic turkeys, susceptibility to LPDV differed between domestic strains, with some having a higher incidence of disease when compared to others (Ianconescu et al 1981). There are six recognized subspecies of wild turkeys in North America, which morphologically differ to varying degrees but are genetically distinct. Each subspecies has its own geographic range, but there is extensive overlap and hybridization between some subspecies. The most widespread subspecies is the Eastern wild turkey whose historic range extends throughout the Eastern half of the US. This subspecies has been the source for many restoration efforts throughout North America, so populations of Eastern wild turkeys or hybrids currently are found throughout the United States (Tapley et al 2004). In this study, the presence of hybrids and the lack of genetic testing precluded a detailed comparison of LPDV infection between subspecies of wild turkey; however, these data suggest that the three subspecies within the region sampled were all susceptible to LPDV infection. To our knowledge, LPDV has not been identified in any wild upland game bird species other than wild turkeys. Considering chickens, domestic turkeys, and wild turkeys have all been shown to be permissive hosts for LPDV (Ianconescu et al 1983; Allison et al 2013) and the overlap between wild turkeys and other wild galliforms, additional LPDV testing of wild upland game bird species is warranted, including Northern bobwhite quail

(*Colinusvirginianus*),ring-necked pheasants, chukar partridge (*Alectorischukar*), and prairie chickens.

As this surveillance effort relied on field biologists and hunters for sample collection, efforts were made to focus on tissues that were both easy to identify and collect and that were likely to be positive based on experience (Allison et al, 2013) and known viral tropism (lymphoid tissues; Biggs 1997). Depending on the state, samples of bone marrow, spleen, and/or liver were collected. Based on paired sampling of a subset of hunter-killed turkeys and diagnostic cases, bone marrow was the best tissue to identify LPDV proviral DNA in infected birds. The extent of differences in LPDV detection between hunter-killed turkeys and diagnostic cases may be due to the handling of the tissues and subsequent post-mortem condition. The turkey carcasses of diagnostic cases were more likely to be maintained under appropriate cold chain until they reach the diagnostic laboratory, where necropsies were performed immediately and the samples processed for LPDV testing. While attempts were made to maintain the cold chain for hunter-killed turkey samples, the tissues likely went through multiple transfers and varying storage or handling conditions prior to reaching the laboratory for processing. Subjectively, based on gross appearance, bone marrow, contained within the tibiotarsus, appeared to withstand autolysis more than the spleen or liver (J. Brown, personal communication). In addition to being the most sensitive tissue for LPDV detection, the tibiotarsus is easily identified, collected, and stored, making it a useful sample for wild bird disease surveillance.

The data reported herein represent a large-scale molecular surveillance for LPDV in apparently asymptomatic, hunter-killed wild turkeys. To our knowledge this is the first wild bird survey of its kind for any avian retrovirus. While the inferences that can be made from these

data on LPDV ecology and epidemiology are limited, they certainly support the conclusion that this virus, previously thought to only be a pathogen of domestic turkeys and exotic to North America, is widespread in wild turkeys throughout the Eastern US. Many unknowns associated with LDPV still exist, including the population significance in wild turkeys, the susceptibility of domestic poultry to wild turkey strains of LPDV, and the best means to culture the virus *in vitro*. In regards to the latter, identifying a way to propagate LPDV *in vitro* is a critical step needed for fulfilling Koch's postulates, characterizing the pathobiology of LPDV through challenge studies, and interpreting diagnostic results based on molecular assays.

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Table 3.1. Prevalence of LPDV proviral DNA from hunter-killed wild turkey samples from various states.

STATE	TISSUE TYPE	n	# POSITIVE (% w/ 95% Confidence Interval)
South Carolina	Liver	74	39 (53% ±11%)
West Virginia	Liver	47	26 (55% ±14%)
New York	Liver	7	4 (57%)
	Bone Marrow	266	128 (48%)
	TOTAL	273	132 (48% ±6%)
Virginia	Bone Marrow	59	17 (29% ±12%)
Florida	Liver	171	77 (45% ±7%)
Louisiana	Liver	96	57 (59% ±9.8%)
Oklahoma	Liver	27	7 (26% ±17%)
New Jersey	Bone Marrow	48	22 (46% ±14%)
Missouri	Liver	39	14 (36%)
	Bone Marrow	35	8 (23%)
	TOTAL	74	22 (30% ±10%)
Georgia	Liver	34	12 (35%)
	Bone Marrow	14	8 (57%)
	TOTAL	48	20 (42% ±14%)
New Hampshire	Bone Marrow	30	25 (83% ±13%)
Vermont	Bone Marrow	28	20 (71% ±17%)
Kansas	Liver	5	1 (20%)
	Bone Marrow	18	7 (39%)
	TOTAL	23	8 (35% ±19%)
Massachusetts	Bone Marrow	9	5 (56% ±33%)
Maine	Bone Marrow	61	50 (82% ±10%)
Rhode Island	Bone Marrow	9	3 (33% ±31%)
North Carolina	Liver	7	3 (43%)
	Bone Marrow	76	30 (39%)
	Spleen	4	1 (25%)
	TOTAL	87	34 (39% ±10%)
TOTAL		**1164**	**564 (47%)**

Table 3.2. Regional differences in prevalence of LPDV proviral DNA among hunter-killed wild turkeys

Tissue type	Region*	n	No. positive (%)**
All combined	Northeast	137	103 (75.2)[a]
	MidAtlantic	427	197 (46)[b]
	Southeast	476	227 (47.7)[b]
	Central	124	37 (29.8)[c]
Bone marrow	Northeast	137	103 (75.2)[a]
	MidAtlantic	373	167 (44.8)[b]
	Southeast	90	38 (42.2)[b,c]
	Central	53	15 (28.3)[c]

*States in reach region: Northeast (Maine, Vermont, and New Hampshire), MidAtlantic (New York, New Jersey, Virginia, West Virginia), Southeast (Georgia, Louisiana, South Carolina, Florida, North Carolina), and Central (Oklahoma, Missouri, Kansas)
**Different letters indicate significant differences within two groups [All combined and bone marrow only]

Table 3.3. Results of matched tissue testing of liver, spleen, and bone marrow from diagnostic cases submitted to SCWDS and asymptomatic hunter-killed wild turkeys.

Tissue Tested	Number positive/No. tested	
	Clinical Cases	Hunter-Killed
Liver	19/20[a]	8/15[a]
Spleen	16/20[a]	9/13[a]
Bone Marrow	20/20[a]	15/15[b]

*Different letters indicate significant differences within two groups [CC and HK].

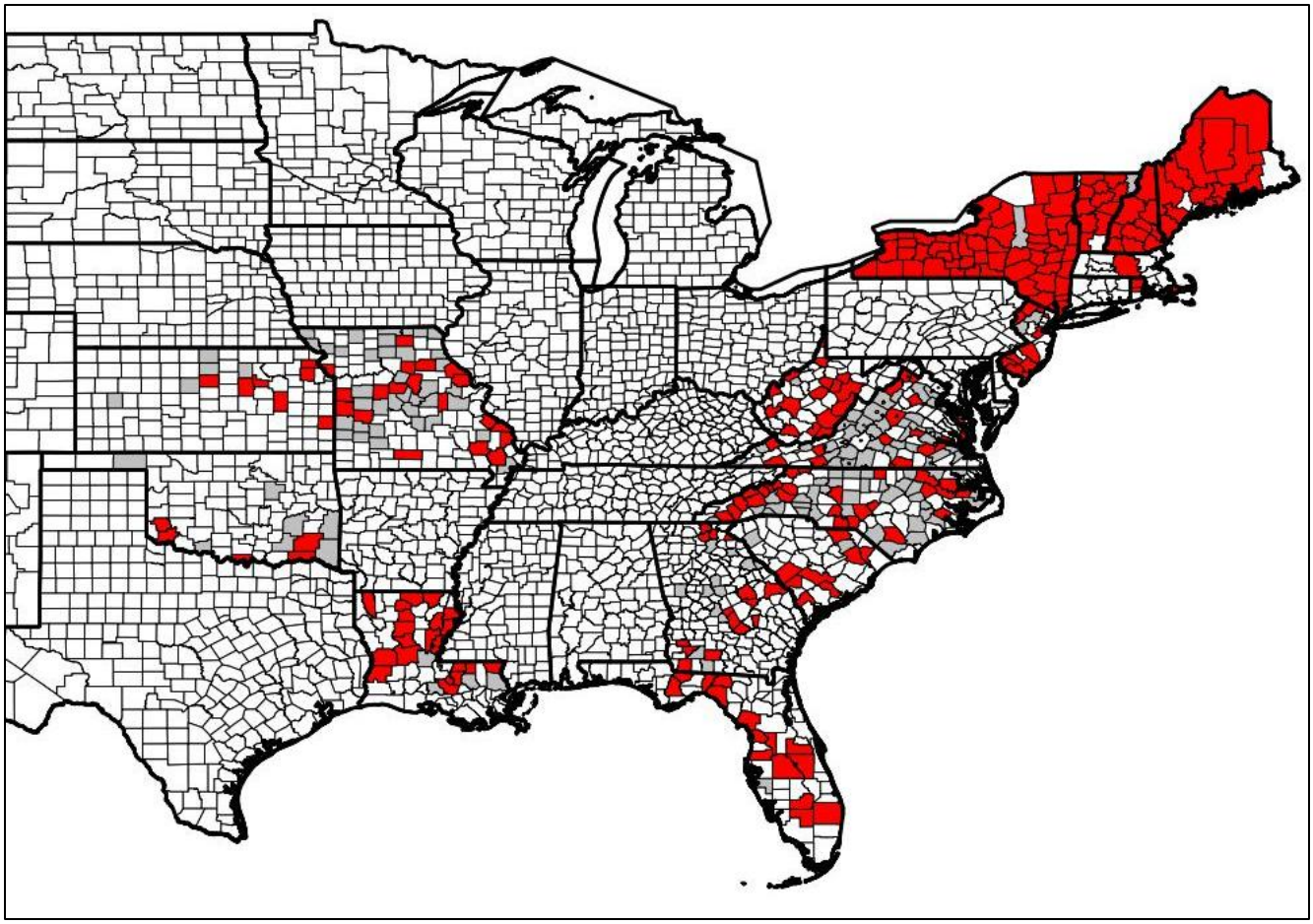

Figure 3.1. Distribution of wild turkey samples testing positive (red) and negative (gray) for LPDV proviral DNA.

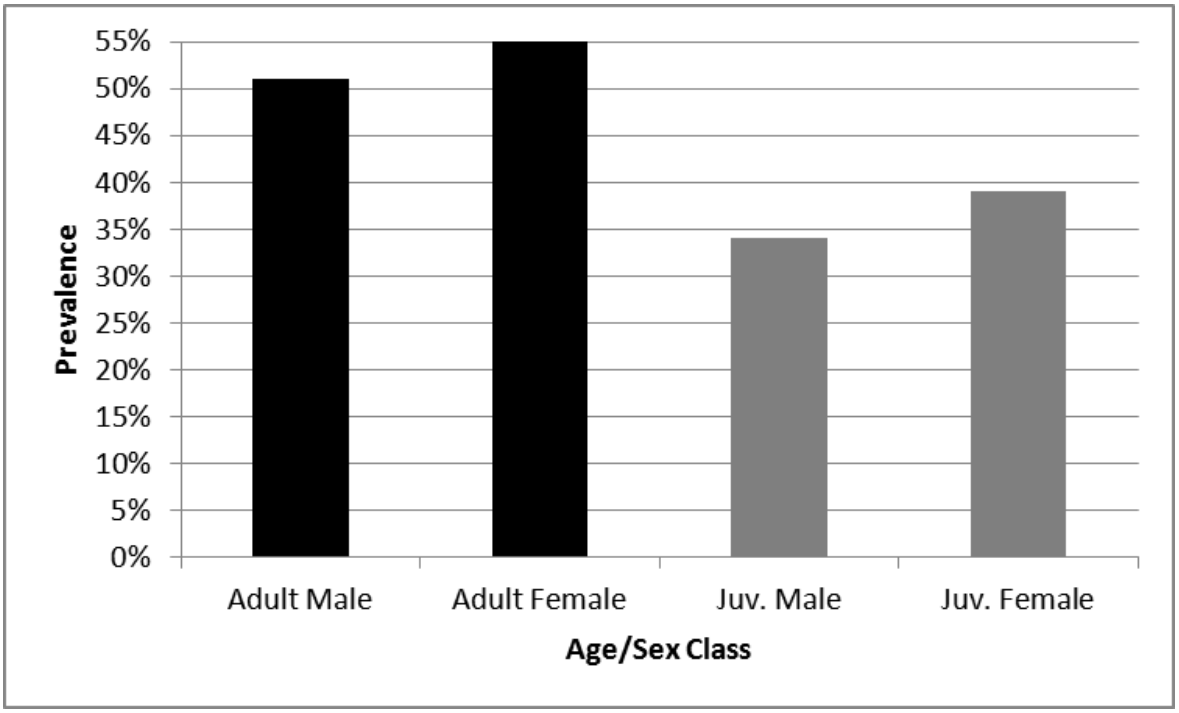

Figure 3.2. Age/Sex differences in prevalence of LPDV proviral DNA across all 17 states sampled.
*Significant difference found between adults vs. juvenile wild turkeys (p=0.0001)
**No significant difference found between males vs. females (p=0.53)

CHAPTER 4

CONCLUSIONS

Lymphoproliferative disease virus (LPDV) has become a pathogen that merits additional research due to the recent reports in wild turkeys in the United States; a finding that supports a novel host record and geographic location. This study looks to further our understanding of LPDV in asymptomatic hunter-killed wild turkeys. Our goals were to: 1) determine the prevalence and geographic distribution of LPDV in wild turkeys in the Eastern U.S. and 2)Determine the most appropriate tissue for molecular detection of LPDV.

Our research concluded that there is a high prevalence of LPDV among healthy wild turkeys throughout the United States with a wide geographic distribution. Based on this surveillance effort, we found that at least three subspecies are susceptible to infection in the United States, including the Eastern, Osceola, and Rio Grande subspecies. We also conclude that bone marrow is the best tissue to detect LPDV proviral DNA by PCR; currently the primary diagnostic tool available.

There is still much to learn regarding LPDV in wild turkeys in the United States, such as the significance this virus has on wild turkey populations, define the epidemiology of LPDV in wild turkeys, and the potential risk of spillover into both commercial and backyard domestic poultry flocks. A major focus should also be placed upon improving diagnostics for LPDV, such as determining a viable way to culture the virus and determine a successful protocol for antemortem diagnostics. Antemortem sampling could be an important tool if translocation efforts are needed.