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RETROVIRAL INFECTION DYNAMICS IN MAINE'S WILD TURKEYS

By

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A DISSERTATION

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

(in Ecology and Environmental Sciences)

The Graduate School

The University of Maine

December 2021

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By Stephanie Shea

Dissertation Advisor: Dr. Pauline Kamath

An Abstract of the Dissertation Presented
in Partial Fulfillment of the Requirements for the
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(in Ecology and Environmental Sciences)
December 2021

Widespread wild turkey reintroductions in the late 1900s have led to increases in population density and geographic distribution across North America. This rapid population expansion has put them into proximity with closely-related wild and domestic avian species, increasing the risks of pathogen transmission. Lymphoproliferative disease virus (LPDV) is an avian oncogenic retrovirus detected in wild turkeys in 2009, and previously known to infect domestic turkeys. Following its initial detection, surveys reported variable LPDV prevalence across eastern North America with most wild turkeys being asymptomatic, however diagnostic cases revealed 10% mortality of LPDV-infected individuals. Given its recent detection, little is known about LPDV ecology, transmission or evolution in wild turkeys. We sought to evaluate (1) an alternative detection method for surveillance, (2) individual risk factors, (3) fitness effects, and (4) the genetic diversity and evolutionary history of LPDV in Maine's wild turkeys. From 2017–2020, we collected tissues and associated data from 72 hunter-harvested and 627 live-captured wild turkeys, and attached radiotransmitters to a subset of live-captured females to monitor survival and reproduction. We used PCR to estimate the infection prevalence of LPDV (59%) and reticuloendotheliosis virus (REV; 16%), another oncogenic retrovirus. In a sample subset, we used plate agglutination to determine the prevalence of exposure to the bacteria,

Mycoplasma gallisepticum (74%) and *Salmonella pullorum* (3.4%). We found cloacal swabs are a reliable LPDV detection method for live-captured wild turkeys. Sex, age, and season were significant predictors of LPDV infection, with females, adults, and individuals sampled in spring having a higher infection risk. Furthermore, we found both LPDV and REV infection negatively affected individual fitness by reducing clutch size and weekly hen survival rate, respectively. Finally, LPDV in Maine is characterized by high diversity and weak spatial genetic structure, which we hypothesize may be driven by high mutation rates, intrahost pathogen dynamics, and/or the history of human-induced and natural wild turkey movement across the state. Overall, this study provides valuable insights into LPDV infection, transmission, and evolution in wild turkeys, data which will aid in future disease monitoring and risk assessments to evaluate effects of infection on wild turkey population dynamics.

DEDICATION

For my mom, Teri Shea, for teaching and encouraging me to reach for the sky, even if I'm afraid of heights. For my dad, Don Shea, for taking me outside and always, always, asking questions. Both of whom are the wind beneath my wings.

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LIST OF ABBREVIATIONS

- Abbreviation 1. DNA – Deoxyribonucleic Acid
- Abbreviation 2. PCR – Polymerase Chain Reaction
- Abbreviation 3. LPDV – Lymphoproliferative disease virus
- Abbreviation 4. REV – Reticuloendotheliosis virus
- Abbreviation 5. WMD – Wildlife Management District

CHAPTER 1

DETECTING LYMPHOPROLIFERATIVE DISEASE VIRUS IN WILD TURKEYS USING CLOACAL SWABS

The monitoring of infectious diseases in wildlife populations is crucial for assessing animal health, pathogen range expansion, and the risk of spillover to naive species, but may be resource and labor intensive. Lymphoproliferative disease virus (LPDV) is an avian oncogenic retrovirus that was first identified in wild turkeys (*Meleagris gallopavo*) in 2009, though it historically caused mortality in domestic turkeys in Europe and Israel. Subsequent surveys detected a high prevalence and broad distribution throughout the eastern United States, warranting research to evaluate the effects of LPDV on wild turkey populations. Current LPDV diagnostics require the collection of tissues, such as bone marrow from dead birds or blood during live capture. In this study, we assessed the sensitivity (true positive) and specificity (true negative) of cloacal swab samples as an alternative LPDV detection method. We compared results from cloacal swab samples with both postmortem detection from bone marrow and antemortem detection from blood, using a multi-tube PCR approach with 3 replicates. Swab samples collected from live-captured turkeys had a greater sensitivity (88%) than swabs collected from hunter-harvested turkeys (31%), whereas specificity was similar for both collection approaches (live-capture swabs = 75%, $n = 85$; hunter-harvest swabs = 80%, $n = 54$). In live-captured turkeys, the estimated LPDV prevalence using cloacal swab samples (73%) was not significantly different from the true prevalence determined using coupled blood samples (76%). However, in hunter-harvested turkeys, the estimated prevalence using cloacal swab samples (28%) was different from the true prevalence based on coupled bone marrow samples (72%). In summary, cloacal swab samples can be used to reliably detect LPDV infection in live-

captured wild turkeys, but should not be used for LPDV detection in hunter-harvested wild turkeys.

Introduction

Wild turkeys (*Meleagris gallopavo*) experienced a significant reduction in both abundance and geographic distribution across North America due to over-hunting and land use change beginning in the early 1800s (Aldrich 1967; Kennamer et al. 1992), reaching an ultimate low in the 1930s (Mosby 1975). Successful reintroductions of wild turkeys in the mid-to-late 20th century resulted in an expansion of the species' range, sometimes to areas beyond those inhabited prior to European settlement. Reintroduction efforts and increased population sizes have raised concerns regarding human-wildlife interactions and pathogen spread within wild turkeys, as well as spillover to other wild or domestic animals. A retrospective survey of wild turkey carcasses submitted to a diagnostic lab in Ontario attributed greater than 25% of morbidity or mortality cases to infectious diseases (MacDonald et al. 2016), highlighting the need for routine disease surveillance in wild turkey populations. Disease surveillance has been increasingly recognized by agencies across the country; for example, the Maine Department of Inland Fisheries and Wildlife identified disease monitoring as a top priority in their most recent Big Game Management Plan, which included wild turkeys (MDIFW 2017).

Lymphoproliferative disease virus (LPDV) is an avian oncogenic retrovirus that can cause lymphoid tumors in wild and domestic turkeys (Biggs et al. 1978, Allison et al. 2014). The pathogen was first detected in domestic turkeys in Europe and Israel, often resulting in flock mortality greater than 20% (Biggs et al. 1978; Gazit and Yaniv 1999), and the pathogen has been shown to be capable of infecting chickens in experimental settings (Ianconescu et al. 1983). In 2009, LPDV proviral DNA was first identified in a wild turkey in the United States, and

subsequent surveys revealed a high prevalence (26–83% by state) and broad distribution across the eastern United States (Allison et al. 2014; Thomas et al. 2015; Alger et al. 2017), and Canada (MacDonald et al. 2019a, 2019b). Although surveillance of hunter-harvested wild turkeys suggested 100% of LPDV-infected turkeys may be outwardly asymptomatic (Thomas et al. 2015), other studies have reported neoplastic lesions in ~15% of clinically-ill birds infected with LPDV (Allison et al. 2014). However, coinfection of LPDV with other pathogens was also reported (Allison et al. 2014; MacDonald et al. 2019a, 2019b), raising concerns that LPDV infection may increase susceptibility to other pathogens and induce subsequent disease symptoms. The host range of LPDV within North America is still under investigation as it has not been detected in other upland game bird species or domestic fowl (MacDonald et al. 2019b), and the potential for spillover (i.e., transmission from one species to another) remains unknown. Within wild turkey populations, little is understood about LPDV distribution, population-level demographic impacts, and status as an emerging or previously undetected pathogen. These gaps in our knowledge have prompted the inclusion of LPDV monitoring in wild turkey disease surveillance programs (Allison et al. 2014; MacDonald et al. 2019b).

Current sampling for LPDV diagnostics relies on sampling of blood from live-captured turkeys (Alger et al. 2015), or collection of tissue from dead turkeys (Thomas et al. 2015). The blood's separated buffy coat layer (white blood cells) is the standard sample type for antemortem detection of LPDV in a genetic-based assay (Alger et al. 2015), whereas bone marrow is typically used for postmortem detection (Thomas et al. 2015). The use of whole blood has been shown to be comparable, based on sensitivity (true positive) and specificity (true negative), to the buffy coat layer (97% sensitivity, 100% specificity) and bone marrow (100% sensitivity, 89% specificity) for LPDV detection (Alger et al. 2015).

Refinement to disease sampling methods can improve the welfare of wild turkeys during and following capture. Increased handling time, in particular, has been associated with post-capture mortality in wild turkeys (Nicholson et al. 2000). Wild turkeys are often captured during winter months when food resources are low, and northern populations in particular are exposed to extremely cold temperatures. Severe winter conditions may increase stress levels (Brown 1961), contribute to overwinter mortality (Kane et al. 2007), negatively affect body condition, and reduce productivity of turkey hens (Porter et al. 1983). Live-capture of wild turkeys is considered invasive (Whatley et al. 1977), and multiple simultaneous stressors can have an additive negative effect on survival, which has been demonstrated in laboratory settings (McFarlane et al. 1989; Miller 1990). Therefore, protocols for wild turkey sample collection during live-capture should be refined to reduce stress, while still maintaining diagnostic accuracy.

Cloacal swab samples are an appealing alternative sample type for LPDV diagnostics. Some permitting agencies or institutional animal care and use committees (IACUCs) may require additional justification to allow blood draw, and less invasive cloacal swabs may offer an alternative that refines the sampling procedure (NRC 2011) to improve animal welfare. During live-capture, cloacal swab samples can be collected alongside other tasks, such as attaching leg bands in order to reduce handling time. Drawing blood safely and effectively requires safety measures, training, and experience, and alternative, less specialized methods such as cloacal swab sampling could facilitate sample collection by a greater number of field personnel. This is especially true during winter months, when freezing temperatures may result in numbness and/or sensory loss (Reamy 1998), which can affect safe handling of needles during blood collection. In addition to live-captured individuals, cloacal swab sampling may contribute to LPDV

surveillance in hunter-harvested birds. While cloacal swab sampling may prove to be a useful alternative, the accuracy of this sample type for LPDV detection has yet to be investigated.

The goal of our study was to evaluate the diagnostic sensitivity and specificity of cloacal swab samples as an efficient sample type for the detection of LPDV in comparison with current diagnostics based on blood or bone marrow. Furthermore, we aimed to identify whether cloacal swab samples could be used to provide an accurate estimate of LPDV prevalence in the wild turkey population. We collected cloacal swab samples, paired with either blood or bone marrow, to compare cloacal swab sample accuracy between the respective collection methods (i.e., live-captured versus hunter-harvested).

Methods

Study Area and Sample Collection

During 2017 and 2018, we collected 139 cloacal swab samples across central and southern Maine (Figure 1.1). Swab samples were coupled with either bone marrow ($n = 54$) or blood samples ($n = 85$) from hunter-harvested or live-captured wild turkeys, respectively. We collected dry cloacal swab samples from live and dead turkeys by inserting a sterile polyester swab into the cloaca, rotating several times, and placing the entire swab in a sterile 15-mL collection tube without media. We stored samples at -80°C until DNA extraction.

We collected the tarsometatarsus (leg) bone from hunter-harvested turkeys during the wild turkey hunting season in May of 2017 and 2018, using loppers that were sterilized with 70% ethanol between the processing of each sample. The tarsometatarsus was kept on ice in the field. We asked hunters to report the time of harvest (in Maine, hunters have 18 hours to report harvests) in order to calculate the time delay between harvest and sampling, which ranged 20–261 minutes.

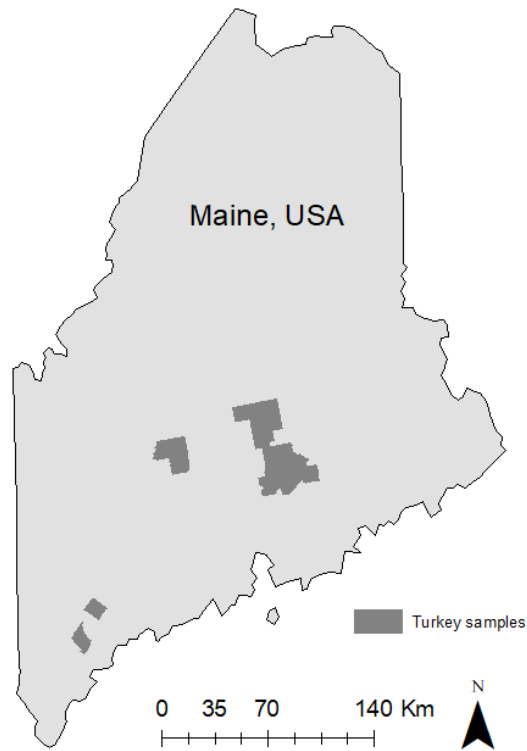


Figure 1.1 Study area of sampled wild turkeys in Maine identified to the town level. Wild turkeys were sampled across central and southern Maine towns to evaluate cloacal swabs as a method for lymphoproliferative disease virus detection in 2017 and 2018.

In the lab, we extracted bone marrow, the standard postmortem sample type for LPDV detection (Thomas et al. 2015), from the leg bone; we used loppers to expose the marrow and transferred the marrow with tweezers to a 2-mL tube. Loppers, sterilized using 70% ethanol and flame from a Bunsen burner, were used to expose the marrow and tweezers, sterilized at 250°C for at least 1 minute with a Germinator 500 Bead Sterilizer (Cell Point Scientific, Gaithersburg, MD).

We employed rocket nets to capture live wild turkeys from January through March 2018 and drew blood from the brachial vein into ethylenediaminetetraacetic acid (EDTA) collection tubes. We centrifuged the blood for 15 minutes at 2500 RPM to optimize collection of the buffy coat layer, which consists of the isolated white blood cells and is the standard antemortem sample type for LPDV detection (Alger et al. 2015). For 2 (out of 85) samples, blood collection volume was low (approximately <1-mL) and, thus, we vortexed the collection tube and took a whole blood sample, which has been shown to yield results that are comparable to detection based on buffy coat alone (97% sensitivity and 100% specificity; Alger et al. 2015). Hereafter, we considered the whole blood and buffy coat samples collectively as blood. All capture, handling, and sampling of wild turkeys was approved by the University of Maine Institutional Animal Care and Use Committee (IACUC Protocol # A2017_11_03).

DNA Extraction and PCR

We extracted genomic DNA from bone marrow, blood, and cloacal swab samples using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Cloacal swab samples required additional steps to ensure DNA material was dislodged from the swabs. First, we allowed the swabs to thaw upright in their original 15-mL collection tube before transferring the tips to a 2-mL tube. We sterilized scissors and forceps used for transferring swabs with 70% ethanol between samples. Next, we added ATL buffer to each sample and mixed contents thoroughly using the TissueLyser II (Qiagen, Valencia, CA) for 30 seconds at 25 Hz. From this point forward, we followed the manufacturer's protocol for animal tissues. We included a negative control for each extraction, with the addition of a sterile swab in all swab extractions. We quantified the DNA concentration of each extraction using a NanoDrop-1000 Spectrophotometer or Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA).

A 413 base pair region of the retroviral *gag* gene in LPDV was amplified through PCR using the following primer sequences: LPDV-F 5'-ATGAGGACTTGTTAGATTGGTTAC-3', and LPDV-R 5'-TGATGGCGTCAGGGCTATTTG-3' (Allison et al. 2014). All PCR reactions were carried out in a total volume of 25- μ L, using the following reagent concentrations: 0.6–1,268.0 ng DNA (from blood, bone marrow, or cloacal swab samples), 0.2 μ M primers (Integrated DNA Technologies, Coralville, IA), 1.5 mM MgCl₂ (Promega, Madison, WI), 0.2 mM dNTPs (New England Biolabs, Ipswich, MA; Promega, Madison, WI), 0.625 units of GoTaq DNA Polymerase (Promega, Madison, WI), and buffer (Promega, Madison, WI). PCR cycling conditions for DNA extracted from blood involved an initial denaturation at 95°C for 3 minutes, followed by 34 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 68°C for 1 minute, and ended with a final elongation step for 5 minutes at 68°C (Alger et al. 2015). For cloacal swab samples, we used a multi-tube approach with 3 total PCR replicates per sample, and increased the cycles to 40. For bone marrow, PCR cycling conditions involved an initial denaturation at 94°C for 2 minutes, followed by 44 cycles of 94°C for 45 seconds, 50°C for 1 minute, and 72°C for 1 minute, and ended with a final elongation step for 2 minutes at 72°C. The negative control for each PCR run was sterile water and the positive control was a known LPDV-positive sample, which was confirmed through sequencing. Amplification of the target region was assessed by electrophoresis, using a 1% agarose gel, and visualized with an Azure c150 Imaging System (Azure Biosystems, Dublin, CA). For further confirmation, all positive PCR products were enzymatically cleaned using Exonuclease 1 and Shrimp Alkaline Phosphatase (ExoSAP-IT; Applied Biosystems, Foster, CA), and sequenced, using the primers listed above, on a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) at the University of Maine DNA Sequencing Facility.

Statistical Analysis

We evaluated the diagnostic sensitivity and specificity of cloacal swab samples for LPDV detection, compared to blood or bone marrow. We considered wild turkeys to be infected with LPDV if at least 1 of the 3 cloacal swab sample replicates returned a PCR-positive result. In contrast, we determined turkeys to be uninfected if all 3 cloacal swab sample replicates were PCR-negative. All analyses used the pooled results from the 3 replicates. We used the LPDV diagnostic results from blood or bone marrow (i.e., herein standard sample types) as the measure of true infection status (Alger et al. 2015, Thomas et al. 2015). We referred to a positive result from the swab sample-based PCR assay as a true positive if a positive result was also obtained from the PCR assay using the paired standard sample type. Likewise, we considered a negative result from the swab sample-based PCR assay that matched a negative result from the assay using the paired standard sample type as a true negative (see Table 1.1 and 1.2 for examples).

Table 1.1 Lymphoproliferative disease virus diagnostic results in hunter-harvested wild turkeys in Maine, based on a PCR-assay using bone marrow (BM) and cloacal swab (Swab) samples collected in May of 2017 and 2018. Cloacal swab sample results were determined through 3 pooled PCR replicates.

	BM (+)	BM (-)	Total
Swab (+)	12 ^a	3 ^b	15
Swab (-)	27 ^c	12 ^d	39
Total	39	15	54

^aTrue positive; ^bFalse positive; ^cFalse negative; ^dTrue negative.

Table 1.2. Lymphoproliferative disease virus diagnostic results in live-captured wild turkeys in Maine, based on a PCR-assay using blood and cloacal swab (Swab) samples collected from January through March 2018. Cloacal swab sample results were determined through 3 pooled PCR replicates.

	Blood (+)	Blood (-)	Total
Swab (+)	57 ^a	5 ^b	62
Swab (-)	8 ^c	15 ^d	23
Total	65	20	85

^aTrue positive; ^bFalse positive; ^cFalse negative; ^dTrue negative.

Alternatively, a false result occurred when there was a mismatch in the results of the assays between the swab sample and the paired standard sample type. For example, a negative swab sample result and a positive standard sample result was considered to be a false negative, whereas a positive swab sample result and a negative standard sample result was a false positive (see Table 1.1 and 1.2 for examples). Using these definitions, we calculated apparent prevalence, true prevalence, positive predictive value (PPV), and negative predictive value (NPV; Table 1.3).

We used the epiR package (Stevenson et al. 2019) to obtain an estimate for Cohen’s Kappa (Kappa; Cohen 1960, McHugh 2012), which signifies the level of agreement beyond chance between 2 sets of binary variables. The Kappa statistic is ranked from 0–1 with 0 indicating agreement is equivalent to chance and 1 indicating perfect agreement, described as an increasing spectrum of slight, fair, moderate, and substantial agreement (Cohen 1960; McHugh 2012). We then performed a Z-test to determine if the Kappa result (measure of agreement) was statistically significant. Additionally, we performed a McNemar test to assess whether there was

Table 1.3. Metrics evaluating the detection of lymphoproliferative disease virus in Maine during 2017 and 2018 using cloacal swabs samples, compared with blood and bone marrow from live-captured or hunter-harvested wild turkeys, respectively.

Metric	Live-captured	Hunter-harvested
Sample size	85	54
Sensitivity	88%	31%
Specificity	75%	80%
Apparent prevalence	73%	28%
True prevalence	76%	72%
Positive predictive value	92%	80%
Negative predictive value	65%	31%

a systematic difference between the prevalence estimated from cloacal swab samples and prevalence estimated from the standard sample types (a change in proportion for the paired data). We performed these analyses comparing results individually for each collection method (live-captured and hunter-harvested) with those from their associated standard sample type.

We evaluated factors that may influence the sensitivity of LPDV detection using swab samples. For each collection method, we ran a generalized linear model (GLM) with a logit link, using both the linear and squared PCR DNA concentration as the independent variables and the ability to detect a positive as the dependent variable to assess the relationship between DNA concentration and LPDV detection probability. For the hunter-harvested collection method, we also ran a GLM with a logit link using the time (minutes) between harvest and sample collection

as the independent variable and the ability to detect a positive as the dependent variable to evaluate whether time from harvest to sampling had an effect on the assay sensitivity.

We performed a 2-tailed Z-test with Yate's continuity correction to test the null hypothesis that the sensitivity and specificity of cloacal swab samples compared with the standard sample types were not significantly different between live-capture and hunter-harvest collection methods. When a significant difference in sensitivity was detected, we ran an additional 1-tailed Z-test to identify which collection method resulted in greater sensitivity. The level of significance for statistical analyses was set at $\alpha < 0.05$. All analyses were performed in RStudio (Version 1.2.5019) using Program R (R Core Team 2019).

Results

Cloacal swab samples collected from hunter-harvested wild turkeys ($n = 54$) had a diagnostic sensitivity of 31% and specificity of 80% compared with bone marrow. Cloacal swab samples collected from live-captured wild turkeys ($n = 85$) had a diagnostic sensitivity of 88% and specificity of 75% compared with blood (Table 3). Swab samples collected during live-capture showed a moderate level of agreement ($k = 0.60$, 0.38–0.81 95% CI), beyond agreement due to chance, with coupled blood samples. A Z-test on the kappa statistic revealed that the Kappa statistic was different from 0 ($Z = 5.52$, $P < 0.001$). The McNemar test (McNemar = 0.69, $P = 0.41$) indicated that there was not a significant difference in prevalence based on cloacal swab samples (73%) and blood (76%) collected from live-captured wild turkeys. When comparing swab samples collected from hunter-harvested wild turkeys, we did not detect agreement ($k = 0.07$, -0.11–0.25 95% CI), beyond agreement due to chance, with coupled bone marrow samples. A Z-test confirmed that the Kappa statistic was not different from 0 ($Z = 0.79$, $P = 0.21$) and the McNemar test (McNemar = 19.20, $P < 0.001$) indicated that there was a

significant difference in prevalence based on cloacal swab samples (28%) and bone marrow (72%) collected from hunter-harvested wild turkeys. For hunter-harvested wild turkeys, the amount of time (minutes) between harvest and cloacal swab sample collection was not a predictor of the ability for cloacal swab samples to detect an LPDV-positive individual ($\beta = -0.01, -0.04 - -0.00$ 95% CI, $n = 39, P = 0.12$).

There was a significant quadratic relationship between PCR DNA concentration (ng/ μ L) and the ability for cloacal swab samples to detect an LPDV-positive individual in hunter-harvested wild turkeys ($\beta = -0.17, -0.34 - -0.05, n = 39, P = 0.02$). For live-captured wild turkeys, the relationship was not significant ($\beta = -0.19, -0.45 - -0.03$ 95% CI, $n = 65, P = 0.06$). The lowest and highest cloacal swab sample PCR DNA concentration to detect a positive from hunter-harvested wild turkeys was 0.60 ng/ μ L and 8.80 ng/ μ L, respectively, whereas the full range of PCR DNA concentrations used in analysis was 0.10–11.12 ng/ μ L. For live-captured wild turkeys, the lowest and highest cloacal swab sample PCR DNA concentration to detect a positive was 0.02 ng/ μ L and 7.68 ng/ μ L, respectively, whereas the full range of PCR DNA concentrations in analysis was 0.02–9.60 ng/ μ L. When using cloacal swab samples from hunter-harvested wild turkeys, maximum assay sensitivity was achieved with a PCR DNA concentration of 4.44 ng/ μ L, with 28% (11/39) of reactions greater than this concentration. Similarly, when using swab samples from live-captured wild turkeys, the DNA concentration at maximum assay sensitivity was 3.94 ng/ μ L, though only 5% (3/65) of reactions were greater than the DNA concentration at maximum sensitivity.

A 2-tailed Z-test ($\chi^2_1 = 32.87, n = 139, P < 0.001$) indicated a significant difference in the sensitivity of cloacal swab samples (compared with the standard sample types) collected from live-captured than for hunter-harvested turkeys. A 1-tailed Z-test revealed that the live-capture

collection method had greater sensitivity than the hunter-harvested collection method, 88% compared with 31% ($\chi^2_1 = 32.87, n = 139, P < 0.001$; Table 3). The specificities of 75% and 80% between the live-capture and hunter-harvested collection methods, respectively, were not different ($\chi^2_1 < 0.001, n = 139, P = 1.00$; Table 3).

We calculated positive and negative predictive values separately for swab samples collected from live-captured and hunter-harvested wild turkeys. Swab samples from live-captured turkeys had a greater PPV (92%) than samples from hunter-harvested turkeys (80%). Similarly, samples collected from live-captured turkeys had a greater NPV (65%) than samples from hunter-harvested turkeys (31%; Table 3).

Discussion

To our knowledge, this is the first confirmation that cloacal swab samples can be used to detect LPDV proviral DNA in wild turkeys. Cloacal swab samples provide a relatively accurate method (88% sensitivity, 75% specificity) for estimating viral prevalence in live-captured wild turkeys during winter months (swabs = 73%, blood = 76%), and may offer a less invasive, more time efficient alternative to current methods, with less needed safety measures and personnel experience than drawing blood. Live-capture and handling can impose stress on the animal and may reduce post-release survival (Nicholson et al. 2000). Hence, the collection of cloacal swab samples (over blood) can reduce handling time, which may improve capture outcomes.

Cloacal swab samples collected from live-captured wild turkeys did not provide perfect accuracy of detection, with 9% of samples yielding false negative results and 6% yielding false positive results. Importantly, we assumed bone marrow and blood have a detection rate of 100%, and although LPDV results based on these sample types has been shown to be very similar (Alger et al. 2015), the true detection probability remains unknown. However, genetic

sequencing of positive products from the swab-based PCR assay indicated that swab samples detected LPDV in 2 individuals (1 live-captured and 1 hunter-harvested) that were considered LPDV-negative according to the standard sample type. This suggests imperfect detection probability of the assays using standard sample types, and therefore the diagnostic sensitivity of using cloacal swab samples for LPDV detection could be slightly underestimated. Thus, we suggest that cloacal swab sampling may be a useful alternative (to blood collection) if funding is not available for the necessary equipment, experienced personnel are not available, cold temperatures affect the safe handling of needles, and/or sampling procedures require refinement to reduce handling time and improve animal welfare. Given that wild turkeys experience multiple stressors during capture that can affect survival, it is important to optimize sampling methods to reduce capture-related impacts.

In contrast to cloacal swab samples collected from live wild turkeys, swab samples from hunter-harvested birds during the spring hunting season were not a useful alternative to bone marrow for LPDV detection postmortem, due to generally low accuracy (31% sensitivity, 80% specificity) in estimating viral prevalence (swabs = 28%, bone marrow = 72%). Other viruses have successfully been detected through postmortem cloacal swab sample collection; for example, cloacal swabs were considered to be a low-resource approach for West Nile virus detection, with a high sensitivity of 95% when compared with the use of brain tissue (Komar et al. 2002). Alternatively, Docherty et al. (2004) observed a lower prevalence of West Nile virus when using cloacal swab samples (38%) compared with feather pulp (77%) samples from avian carcasses ($n = 65$). However, these previous studies differ from ours, because they targeted viral RNA, rather than proviral DNA. In general, research on postmortem viral detection via cloacal

swabs appears to be scarce; this may be due to an inability to detect pathogens in cloacal swab samples collected postmortem, or due to the ease of obtaining tissue samples from carcasses.

The observed difference in the probability of detecting LPDV from swabs in hunter-harvested and live-captured birds could be explained by several factors, including whether sample collection was ante- or postmortem, the month of collection, and/or sample size. Our sampling design did not allow us to disentangle the first 2 factors, given cloacal swab samples from live-captured birds were collected in winter (January–March) and those from hunter-harvested birds were collected in spring (May). Further work is warranted to determine whether season is a factor influencing discrepancies between detection rates, which could be accomplished by obtaining samples from both live-captured and hunter-harvested wild turkeys during the same season. Additionally, increasing sample sizes from hunter-harvested and live-captured wild turkeys could increase the accuracy of the diagnostic sensitivity and specificity estimates.

Furthermore, the delay between the time a turkey was harvested and when cloacal swab sampling occurred may have affected our ability to detect LPDV given that DNA can degrade over time (Johnson and Ferris 2002). Although we documented an apparent negative relationship between time since harvest and LPDV detection probability, this result was not significant. However, the effect of time between harvest and sampling on LPDV detection probability warrants further investigation with larger sample sizes over longer and more evenly distributed time delays.

Another factor that can affect the detection probability of PCR procedures is the DNA concentration used in the assay. There is typically a minimum DNA concentration required to detect a positive, whereas high quantities can inhibit PCR procedures (Maddocks and Jenkins

2017). In hunter-harvested turkeys, we observed PCR sensitivity dropping off at both low and high DNA concentrations, which was reflected in the quadratic relationship documented between DNA concentration and cloacal swab sample PCR assay sensitivity. In contrast, the model evaluating the relationship between DNA concentration and PCR sensitivity in live-captured turkeys was not significant. Variation in DNA concentration as a predictor of PCR assay sensitivity could explain the discrepancy between the diagnostic sensitivity of the 2 collection methods, but the underlying cause of the difference in diagnostic sensitivity requires further attention. The range of PCR DNA concentration was similar for both collection methods used in this analysis (hunter-harvested: 0.10–11.12 ng/ μ L; live-captured: 0.02–9.60 ng/ μ L), but the concentrations from hunter-harvested samples were more evenly distributed across their range (28% of samples greater than the maximum sensitivity value of 4.4 ng/ μ L) than the reactions from live-captured samples (5% greater than the maximum sensitivity value of 3.94 ng/ μ L). The range and distribution of DNA concentration (and its quality) should be standardized to allow direct comparison between the 2 collection methods to fully understand the effect on detection probability.

We confirmed that LPDV proviral DNA is detectable in the cloaca, and future research, including experimental infection, is needed to optimize diagnostic approaches to shed light on patterns of LPDV infection. Proviral DNA indicates infection, but does not distinguish between past and current infection because it is specifically detecting viral integration into the host genome (Payne 1992). Therefore, detection of viral RNA through RT-PCR would be an optimal next step to characterize replication and shedding patterns to identify LPDV transmission dynamics in wild turkeys, which may require cloacal swab samples if shedding occurs through the gastrointestinal tract.

Management Implications

Cloacal swabs can be used as an alternative accurate collection method for LPDV proviral DNA detection in live-captured wild turkeys but should not be used as a detection method in hunter-harvested wild turkeys. Swabs are minimally invasive and more efficient, requiring less equipment, safety measures, and personnel expertise than current diagnostics that rely on blood collection. Further research is needed to determine the effect of season, turkey status (live or dead), PCR DNA concentration, and/or a time delay between harvest and postmortem sampling on LPDV detection probability in order to understand the discrepancy in accuracy between swab sampling from live and hunter-harvested birds. Lymphoproliferative disease virus detection in cloacal swab samples from live birds supports its widespread addition to live-capture protocols, particularly when sampling procedures require refinement, for example if there is a desire to reduce equipment, handling time, safety concerns, and/or expertise requirements.

CHAPTER 2

PATHOGEN SURVEY AND PREDICTORS OF LYMPHOPROLIFERATIVE DISEASE VIRUS INFECTION IN WILD TURKEYS

Growing populations of wild turkeys (*Meleagris gallopavo*), due to reintroduction campaigns, may result in increased disease transmission among wildlife and spillover to poultry. Lymphoproliferative disease virus (LPDV) is a recently discovered avian retrovirus that is widespread in wild turkeys in eastern North America, and may influence mortality and parasite coinfections. Here, we aimed to identify individual and spatial risk factors of LPDV in Maine's wild turkeys. We also surveyed for reticuloendotheliosis virus (REV), *Mycoplasma gallisepticum* and *Salmonella pullorum* coinfections, to estimate trends in prevalence and examine covariance with LPDV. From 2017–2020 we sampled tissues from hunter-harvested ($n = 72$) and live-captured ($n = 627$) wild turkeys, in spring and winter, respectively, for PCR diagnostics of LPDV and REV. In a subset of captured individuals ($n = 235$), we estimated seroprevalence of the bacteria *M. gallisepticum* and *S. pullorum* using a plate agglutination test. Infection prevalence for LPDV and REV was 59% and 16%, respectively, with a coinfection rate of 10%. Seroprevalence of *M. gallisepticum* and *S. pullorum* was 74% and 3.4%, with LPDV coinfection rates of 51% and 2.6%, respectively. Infection with LPDV, and seroprevalence of *M. gallisepticum* and *S. pullorum* decreased and REV infection increased between 2018–2020 during winter sampling. Females (64%), adults (72%), and individuals sampled in spring (76%) had a higher risk of LPDV infection than males (47%), juveniles (39%), and individuals sampled in winter (57%), respectively. Furthermore, LPDV infection increased with percent forested cover ($\beta = 0.014 \pm 0.007$) and decreased with percent agriculture cover for juveniles ($\beta = -0.061 \pm 0.018$) sampled in winter. These data enhance our understanding of individual and spatial

predictors of LPDV infection in wild turkeys and aid in assessing the risk to wild turkey populations and poultry operations.

Introduction

Pathogen monitoring in wild populations is necessary to assess animal health, gauge transmission potential to other wild and domestic species, detect emerging pathogens, and evaluate the threat to human health. The need for pathogen monitoring in wild turkey populations (*Meleagris gallopavo*, order Galliformes) is heightened due to the recent and drastic increases in their population density and size following reintroduction efforts. Studies across several host-pathogen systems have shown that pathogen transmission can increase with host density or group size (Arneberg et al. 1998; Rifkin et al. 2012) and wild turkey populations in Maine increased from zero to as many as 60,000 in just 40 years (Allen 2000; Sullivan 2017). Furthermore, wild bird species commonly share pathogens with each other and poultry (Gortázar et al. 2007), as well as with birds in captive breeding facilities (Stewart et al. 2019), raising concern for spillover to species with economical and/or recreational importance (Gortázar et al. 2007). Wild turkeys are valued as a prized game bird and occur in large social groups at the wildlife-domestic animal-human interface. Their high probability of interacting with both humans and poultry highlights the need for pathogen monitoring in this species.

Pathogen infectivity and pathogenicity varies across space, time (Tack et al. 2012), and host species (Brown et al. 2012), and intraspecies individuals experience varying levels of susceptibility and/or exposure (Muma et al. 2006). For instance, *Toxoplasma gondii* seropositivity in wild birds is reportedly dependent upon age and diet, suggesting transmission is related to feeding behavior thereby informing management strategies (Cabezón et al. 2011). Identifying the contribution of host, seasonal, and spatial risk factors to individual infection

heterogeneity can help better understand transmission dynamics (Paull et al. 2012), ultimately aiding in the prediction of timing, location, or individuals affected.

Lymphoproliferative disease virus (LPDV) is an avian oncogenic retrovirus that is known to cause lymphoid tumors and mortality in domestic turkeys (Biggs et al. 1978; Biggs 1997). In 2009, LPDV was identified in wild turkeys in the United States (US), revealing a host and expanded geographic range for the virus (Allison et al. 2014). Subsequent surveys of apparently asymptomatic wild turkeys reported high prevalence (26–83% by state) in the eastern US (Thomas et al. 2015), as well as among provinces in Canada (31-65%; MacDonald et al. 2019b, 2019a). Wild turkeys can experience clinical symptoms suggestive of LPDV infection, including lesions as reported in 15–25% LPDV-infected wild turkeys submitted to diagnostic labs (Allison et al. 2014; MacDonald et al. 2019a). Furthermore, Allison et al. (2014) attributed an observed 10% of LPDV-positive mortalities to LPDV infection.

Reticuloendotheliosis virus (REV), and the bacteria *Mycoplasma gallisepticum*, and *Salmonella pullorum* (*Salmonella enterica* subspecies *enterica* serovar *pullorum*) occur naturally in a wide range of wild bird species (Bullis 1977; Hartup et al. 2000; Ferro et al. 2017) and can spread via asymptomatic carriers, though little is known regarding their impact on wild turkeys. Reticuloendotheliosis virus can cause runting syndrome and induce immunosuppression and tumor growth in poultry (Fadly et al. 2008). *Mycoplasma gallisepticum* is considered to be the cause of one of the most costly diseases (infectious sinusitis) in the commercial poultry industry (Ley and Yoder 1997). *Salmonella pullorum* infection often leads to mortality in young poultry (Boulianne et al. 2013) or reduced reproductive output in adults (Markos and Abdela 2016).

Host pathogenicity and disease outcome (level of morbidity, mortality) is compounded by coinfection, where a single host individual is infected by at least two distinct infectious agents

(Cox 2001). Coinfection, as observed with LPDV and REV (Allison et al. 2014; MacDonald et al. 2019b, 2019a), may be the result of host immunosuppression, where infection by one pathogen increases susceptibility to another; alternatively, both viruses may opportunistically infect individuals under reproductive, nutritional, or parasite-induced stress. For example, increased mortality, growth retardation, and immunosuppression results from coinfection of avian leukosis virus (ALV), another oncogenic retrovirus closely related to LPDV (Chajut et al. 1992; Allison et al. 2014), and REV in poultry (Dong et al. 2014). In contrast, other studies have reported reduced disease severity (e.g., Schürch and Roy 2004), leading to a plea by researchers to integrate pathogen community ecology into disease research to avoid misinterpretations when assessing pathogens in isolation (Cassirer et al. 2018; Hoarau et al. 2020).

We evaluated LPDV risk factors and distribution, and assessed the coinfection status and prevalence of three additional pathogens in Maine's wild turkeys. Our objectives were to (1) determine the prevalence of LPDV, REV, *M. gallisepticum*, and *S. pullorum*, (2) identify individual heterogeneity in host and spatial risk factors for LPDV infection, and (3) evaluate coinfection as a driver of LPDV infection. These data are valuable for understanding pathogen dynamics and informing disease management of wild turkeys.

Materials and Methods

Field Sampling and Data Collection

We collected samples (bones, whole blood) from wild turkeys ($n = 699$) throughout the state of Maine (Figure 2.1). Tarsometatarsus bones ($n = 72$) were collected from hunter-harvested turkeys at check stations during the 2017 and 2018 male-only spring hunting season (April–June). Live wild turkeys ($n = 627$) were trapped using rocket or drop nets for three winters (December/January–March, 2018–2020).

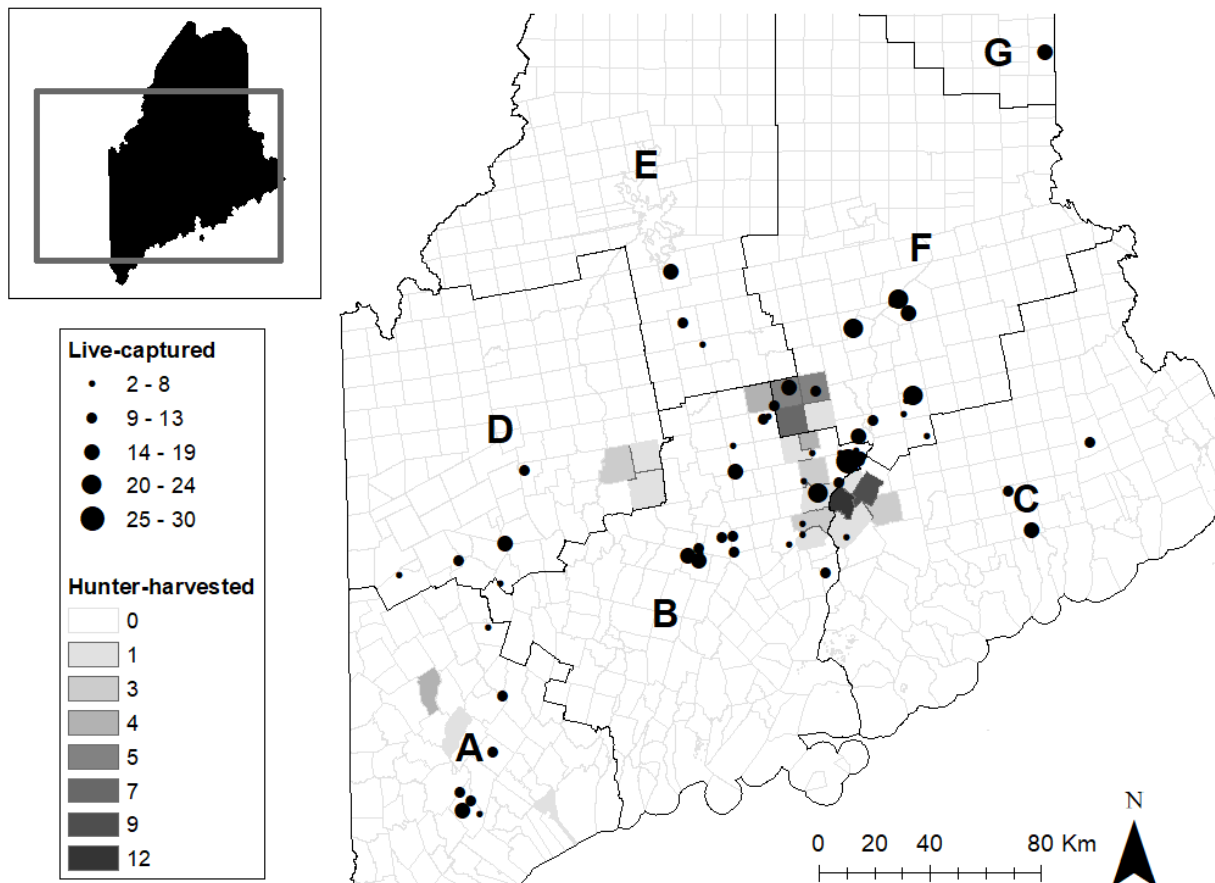


Figure 2.1. Study area of wild turkeys sampled across the state of Maine to determine risk factors of lymphoproliferative disease virus infection from 2017 through 2020. Bureau of Resource Management Administrative Regions (A – F) are outlined. Hunter-harvested individuals are depicted at the town level, with the town of harvest shaded based on sample size from that town. Capture sites are represented by circles sized by the number of live-captured individuals sampled at the site.

Whole blood was collected from live-captured birds for molecular diagnostics of LPDV and REV either: (1) from the brachial vein into an EDTA blood tube (1–5 mL; $n = 263$), or (2) from a foot venipuncture into a capillary tube and stored in queen's lysis buffer (~1mL, $n = 363$). From a portion of individuals, we obtained serum (1–7 mL; $n = 235$) for serological diagnostics of *M. gallisepticum* and *S. pullorum*. In addition, we obtained one bone marrow sample from a live-captured individual post-mortem. All capture, handling, and sampling of wild turkeys was approved by the University of Maine Institutional Animal Care and Use Committee (IACUC Protocol # A2017_11_03).

For all sampled birds, we recorded associated data on the Wildlife Management District (WMD), Maine Department of Inland Fisheries and Wildlife Region (hereafter region), and collection year, and determined sex and age (adult or juvenile; Dickson 1992). For live-captured birds only, we estimated flock size as the number of birds gathering at the bait site.

Sample Processing and DNA Extraction

We extracted bone marrow (post-mortem sample type with highest LPDV detection probability; Thomas et al. 2015) from tarsometatarsus bones using flame-sterilized loppers and tweezers sterilized with a Germinator 500 Bead Sterilizer (Cell Point Scientific, Gaithersburg, MD). For a subset of blood samples ($n = 256$), we centrifuged the EDTA blood tube for 15 minutes at 2500 RPM to isolate the buffy coat layer (ante-mortem sample type with highest LPDV detection probability; Alger et al. 2015). For the remaining blood samples ($n = 370$), whole blood was used for pathogen detection, which has been shown to be comparable to detection from isolated buffy coat (97% sensitivity, 100% specificity; Alger et al. 2015).

We used Qiagen DNeasy Blood and Tissue Kits (Qiagen, Valencia, CA) to extract genomic DNA from blood and tissue samples following manufacturer's instructions. For each

extraction, we included a negative control, and quantified DNA concentration using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) or Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA).

Pathogen Diagnostics

We used molecular and serological approaches for pathogen diagnostics. For LPDV detection, we amplified a 413 base pair region of the retroviral *gag* gene following the PCR cycling conditions described in Alger et al. (2015), using primers described by Allison et al. (2014). All PCR reactions were carried out following the reagent concentrations identified in Shea et al. (*in press*).

For REV detection, we amplified a 580 base pair region of the *pol* gene using primers and cycling conditions described by Bohls et al. (2006). The PCR reaction was carried out in a total volume of 50- μ L, using the following reagent concentrations: 0.6–1,268.0 ng DNA (from blood or bone marrow), 0.2 μ M primers (Integrated DNA Technologies, Coralville, IA), 1.2 mM MgCl₂ (Promega, Madison, WI), 0.2 mM dNTPs (New England Biolabs, Ipswich, MA), 1.25 units of GoTaq DNA Polymerase (Promega, Madison, WI), and 5x buffer (Promega, Madison, WI). In all PCRs, we included a negative (sterile water) and positive control confirmed through sequencing for each pathogen. Amplification of the target region was verified by electrophoresis, using a 1% agarose gel, and visualized with an Azure c150 Imaging System (Azure Biosystems, Dublin, CA).

We used plate agglutination to evaluate *M. gallisepticum* and *S. pullorum* exposure in serum samples ($n = 235$). A drop of antigen (Charles River Laboratories, Wilmington, MA) was mixed with 20 μ L of each serum sample on a glass plate. The plate was agitated, and a sample

was considered positive if aggregation was observed within 2 minutes. Each plate run included a positive (Charles River Laboratories, Wilmington, MA) and negative control (saline).

Estimation of Land Type Usage

We estimated land type percentages within winter home ranges of live-captured individuals to assess the effect of land type on LPDV infection. Our sampling area spanned 13 WMDS (7 in spring and 11 in winter) and all 7 regions (5 in spring and 7 in winter) in Maine (Figure 2.1), representing variation in land types across a gradient of agriculture, developed and forested cover. Using GPS data collected between January 1 and March 15 from a single bird in each flock, we buffered capture site locations by 1.25x (to account for variation in movement) the mean winter home range size using a dynamic Brownian Bridge Movement Model (Kranstauber et al. 2012). We overlaid home ranges with land cover data from the National Land Cover Database (Homer et al. 2015) to estimate land type percentages within each buffer. We believe this is an appropriate assessment of wild turkey land use during winter since their home ranges are known to be smallest during this time of year (Niedzielski et al. 2016).

Pathogen Coinfection and Temporal Trends

We tested for independence of LPDV infection from REV or *M. gallisepticum* using Pearson's Chi-squared test with Yates' continuity correction on contingency tables; $p < 0.05$ indicated lack of independence. We also estimated prevalence by year for each pathogen to illuminate temporal trends. All statistical analyses were conducted in Program R (R Core Team 2021). *Salmonella pullorum* was excluded from this analysis due to low prevalence.

Risk Factors Analysis

We used logistic regression to model relationships between individual and spatial predictor variables and LPDV infection (0 = uninfected, 1 = infected). Using data from both live

and harvested birds, we first ran a univariate model with age as a predictor to confirm previous findings that age is significant factor in LPDV infection (Alger et al. 2017). We additionally ran univariate models to assess potential for bias introduced by differences in capture method (live-capture versus hunter-harvest), season (winter versus spring, respectively), and/or by the sample type (blood versus bone marrow). Because these analyses revealed significant effects of age and season, we performed subsequent analyses by season and partitioned the data by age to identify age interaction terms.

For wild turkeys captured in the winter, we assessed univariate models for all independent variables, including age, sex, REV infection status, density, year, region, WMD, and percent forested, agriculture, and developed cover to determine their relationship with LPDV infection. For harvested turkeys sampled in the spring, sex and land types were excluded because birds were all males and home range data were not available. We used a threshold of $p < 0.25$ for inclusion of single variables or age interaction terms in the initial global model. With this initial global model, we used AICc model selection (R package Aiccmodavg; Mazerolle 2020) to determine which spatial variable, WMD or region, better predicted LPDV infection. We tested for correlation between numeric variables using Pearson's product-moment correlation. Starting with this initial global model, we iteratively removed the variable or interaction term with the highest p -value (McDonald 2009).

Once we arrived upon a final model ($p < 0.15$ for all remaining variables), we added each removed variable back into the model to ensure significance level was not sensitive to the inclusion of other non-significant variables during the selection process (McDonald 2009) and used AICc model selection to ensure the removal of any variable did not result in better model fit. We assessed multicollinearity using the R package regclass (Thompson et al. 2017; Petrie

2020). Finally, we obtained contrasts between each level of multilevel variables using the R package emmeans (Lenth et al. 2021), and calculated odds ratios of contrasts by exponentiating coefficients and confidence intervals or using the R package questionr (Barnier et al. 2020) to calculate directly from the GLM output.

In independent analyses, we followed the same model-building procedure described above, limiting data to individuals with known flock size ($n = 511$) or *M. gallisepticum* status ($n = 235$) to determine their effects on LPDV infection during winter. Furthermore, we partitioned the data by collection year and ran a GLM to determine if month of collection (December–March) influenced LPDV infection in winter.

Results

Pathogen Prevalence, Distribution, and Coinfection

Wild turkey LPDV infection prevalence in Maine was 59% ($n = 699$). Season was a significant predictor of LPDV infection ($p = 0.002$); an individual was 2.5x (1.4–4.5, 95% CI) more likely to be infected in the spring (hunter-harvested; 76%) than in the winter (live-captured; 56%) based on a univariate model, with variation by year (Figure 2.2). Age was significant ($p < 0.001$), with adults 4.1x (3.0–5.6, 95% CI) more likely to be infected than juveniles. These findings support subsequent data partitioning by age within each season subset for risk factor analysis.

We estimated an REV infection prevalence of 16% ($n = 699$), and *M. gallisepticum* and *S. pullorum* seroprevalence of 74% and 3.4%, respectively ($n = 235$), with variation by year (Figure 2.3). Lymphoproliferative disease virus infection was independent from both *M. gallisepticum* ($\chi^2_1 = 0.344$, $n = 235$, $p = 0.56$) and REV ($\chi^2_1 = 1.164$, $n = 699$, $p = 0.28$) status. Dual coinfection rates with LPDV are found in Table 2.1. Twenty-five individuals (11%) were

coinfected with LPDV, REV, and *M. gallisepticum*; four (1.7%) were coinfecting with LPDV, *S. pullorum*, and *M. gallisepticum*.

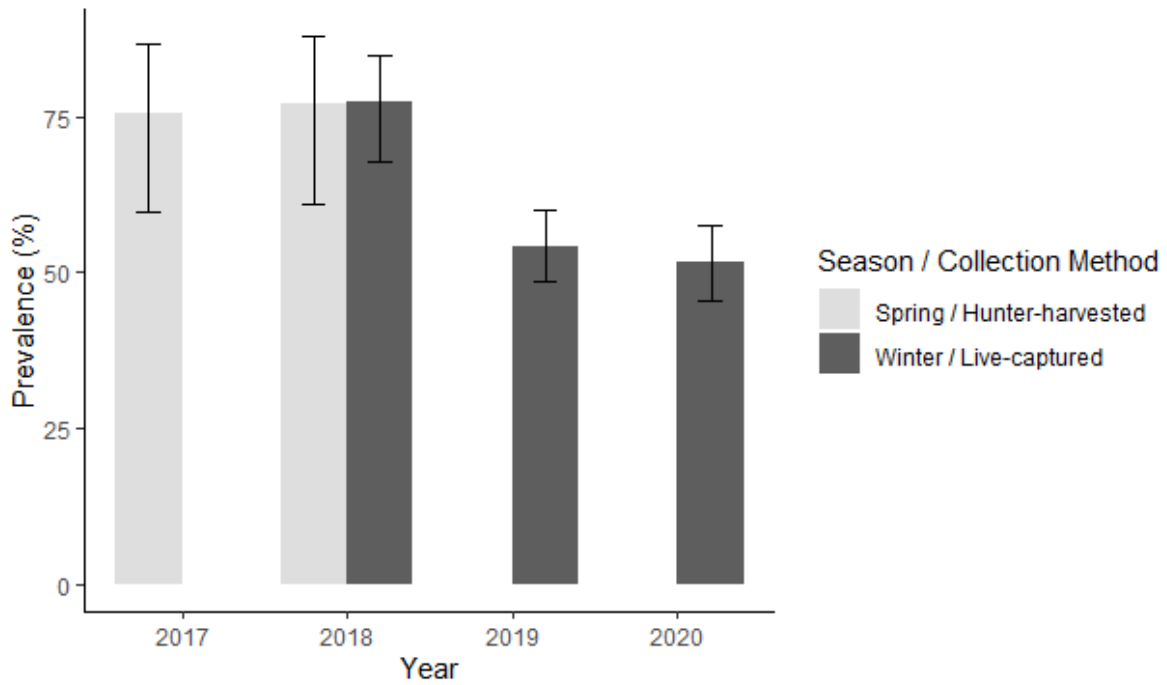


Figure 2.2 Prevalence by year of lymphoproliferative disease virus (LPDV) of live-captured ($n = 627$) or hunter-harvested ($n = 72$) wild turkeys collected in winter or spring, respectively, in Maine. Hunter-harvested individuals were sampled in 2017 and 2018, and live-captured individuals were sampled in 2018 through 2020. Prevalence estimates shown with 95% confidence intervals.

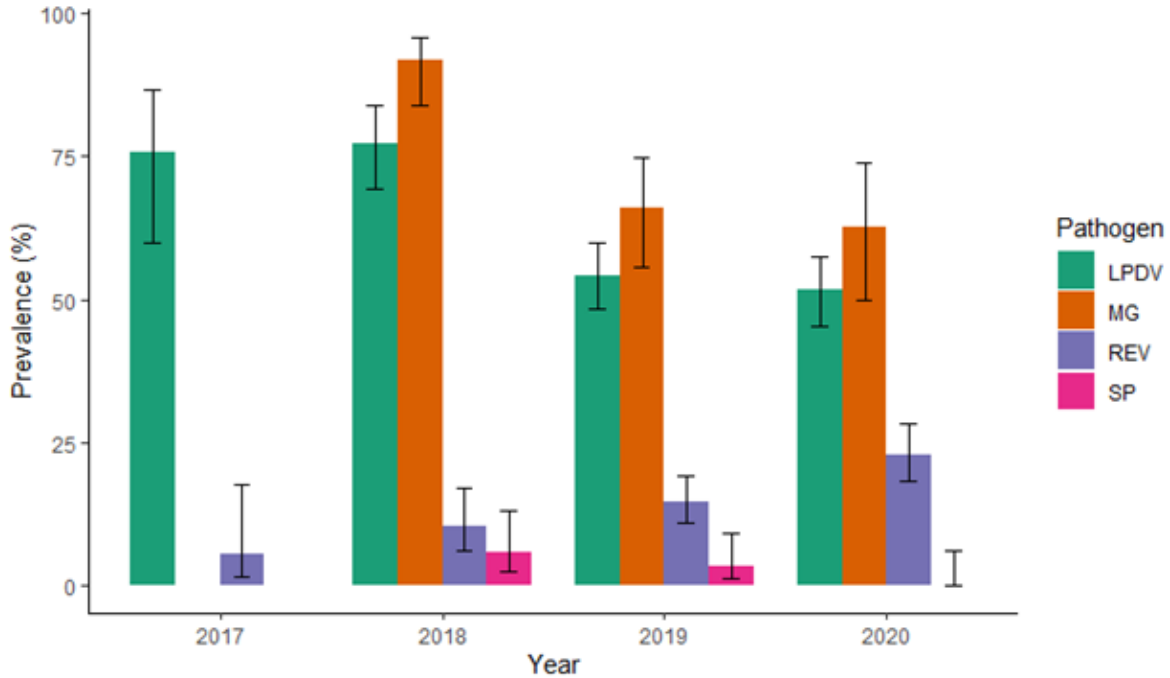


Figure 2.3. Infection prevalence by year of lymphoproliferative disease virus (LPDV; $n = 699$) and reticuloendotheliosis virus (REV; $n = 699$) estimated by molecular diagnostics and exposure prevalence of *Mycoplasma gallisepticum* (MG; $n = 235$) and *Salmonella pullorum* (SP; $n = 235$) estimated using serological procedures in wild turkeys sampled between 2017 and 2020 in Maine. Samples for SP and MG exposure detection were only collected in 2018 through 2020. Prevalence estimates shown with 95% confidence intervals.

Table 2.1 Prevalence of lymphoproliferative disease virus (LPDV) and three co-infecting pathogens (reticuloendotheliosis virus - REV, *Mycoplasma gallisepticum*, *Salmonella pullorum*) in wild turkeys sampled from 2017 through 2020 in Maine.

Pathogen	LPDV	REV	<i>Mycoplasma gallisepticum</i>	<i>Salmonella pullorum</i>
Sample Size	699	699	235	235
# LPDV Positive	NA	409	158	158
# Coinfected with LPDV	NA	73	120	6
Coinfection Prevalence	NA	10%	51%	2.6%

Lymphoproliferative Disease Virus Risk Factors by Season

We sampled a total of 627 (253 juveniles and 374 adults; 351 females and 276 males) live-captured wild turkeys in winter to identify risk factors of LPDV infection. Within each year, collection month during the winter did not significantly predict LPDV infection ($p > 0.20$ for all months). Our final global model best predicting LPDV infection status included region, sex, age, year, percent agriculture and forested cover, and the interaction of age with both agriculture and region (Table 2.2).

Infection with LPDV decreased by year (Tables 2.2 and 2.3; Figure 2.2 and Figure 2.3). The effect of region varied based on age class; there was no difference in LPDV infection between any two regions for adults. In juveniles, LPDV infection was significantly lower in region D compared to regions B and C (Tables 2.2 and 2.3; Figure 2.4).

Table 2.2 Variables included in the final global model predicting lymphoproliferative disease virus in wild turkeys live-captured during winter from 2018 through 2020 in Maine.

Variable^a	Class	Level	Beta ± SE	Odds Ratio (95% CI)	Z value or ratio	p
Sex	NA	Male	-0.412 ± 0.194	0.662 (0.452–0.970)	-2.121	0.034
Year	NA	2018–2020	1.358 ± 0.386	3.888 (1.573–9.583)	3.515	0.001
		2019–2020	0.853 ± 0.231	2.347 (1.366–4.015)	3.693	<0.001
Forested	NA	NA	0.014 ± 0.007	1.014 (1.001–1.028)	2.121	0.034
Age*	Age	Juvenile	-0.061 ± 0.018	0.941 (0.908–0.976)	-3.304	0.001
Agriculture						
Age*	Juvenile	B – D	2.045 ± 0.664	7.729 (1.092 – 54.598)	3.081	0.034
Region		C – D	2.557 ± 0.752	12.897 (1.035–117.919)	3.402	0.012

^aAge, Region, and Agriculture could not be evaluated independently as each one was included in an interaction term in the final global model.

Table 2.3. Prevalence of lymphoproliferative disease virus for categorical variables predicting lymphoproliferative disease virus in wild turkeys live-captured during winter from 2018 through 2020 in Maine.

Variable	Class	Positive	Sample Size	Prevalence	95% CI
Season / Collection Method	Spring / HH ^a	55	72	76.4%	65.4–84.7%
	Winter / LC ^a	354	627	56.5%	52.6–60.3%
Age	Adult	266	374	71.1%	66.3–75.5%
	Juvenile	88	253	34.8%	29.2–40.8%
Sex	Male	129	276	46.7%	40.9–52.6%
	Female	225	351	64.1%	59.0–68.9%
Region (Juvenile)	B	38	110	34.5%	26.3–43.8%
	C	15	23	65.2%	44.9–81.1%
	D	4	22	18.2%	7.3–38.5%
Year	2018	69	89	77.5%	67.8–85.0%
	2019	152	280	54.3%	48.4–60.0%
	2020	133	258	51.6%	45.5–57.6%

^aCollection Method: HH, hunter-harvested; LC, live-captured.

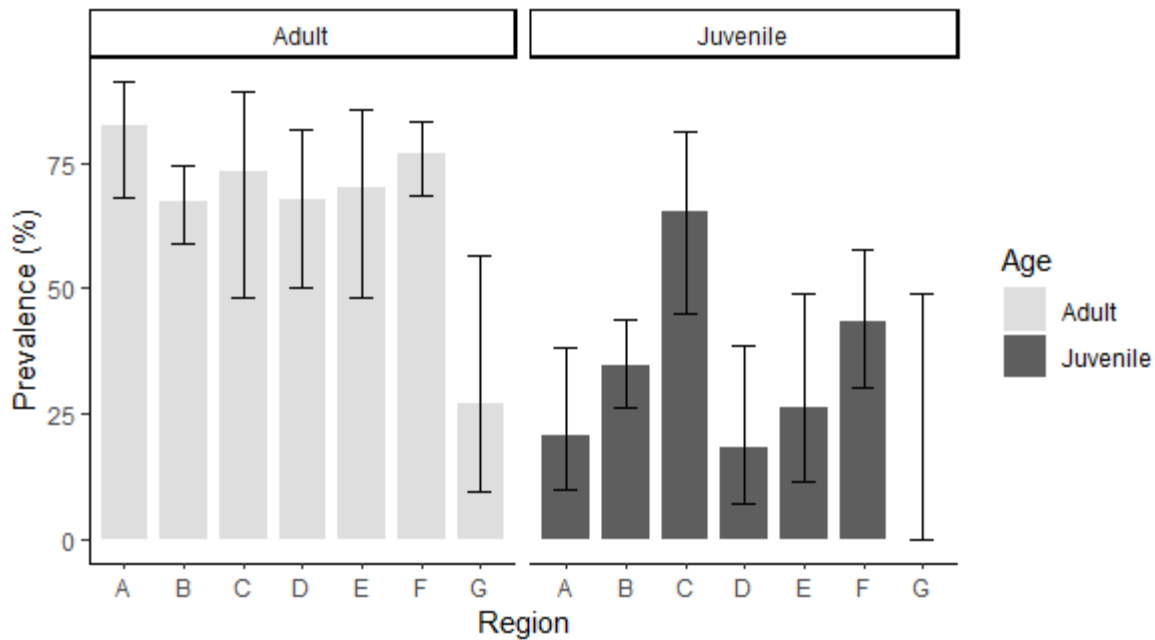


Figure 2.4. Prevalence of lymphoproliferative disease virus by age class and region of collection for wild turkeys live-captured during winter 2018–2020 in Maine. Regions B and C have a significantly higher prevalence than region D for juveniles, while there was no difference in prevalence by region for adults. Prevalence estimates shown with 95% confidence intervals.

Females were 1.5x more likely to be infected than males (Tables 2.2 and 2.3). Percent land type was a significant predictor of LPDV infection; for every 10% increase in percent forested cover, an individual was 10.1x more likely to be infected with LPDV (Tables 2.2 and 2.3). We also found an effect of agriculture on LPDV that varied by age; for every 10% decrease in agricultural cover, a juvenile individual was 10.6x more likely to be infected (Tables 2.2 and 2.3), while no effect was found for adults. Finally, we found no evidence that flock size (mean = 124.7; range = 3–125 birds), REV infection, or *M. gallisepticum* exposure varied with LPDV infection.

We sampled 72 hunter-harvested turkeys (31 juveniles and 41 adults; all males) during spring and determined that age, REV infection status, year of collection, region of collection, and WMD of collection did not influence LPDV infection ($p > 0.15$) and no interaction terms with age fell below the initial inclusion threshold of $p < 0.25$.

Discussion

Lymphoproliferative disease virus is geographically widespread in Maine's wild turkeys, with a prevalence of 59% that decreased from 2017 through 2020. Wild turkeys are also infected with REV (16%) and have been exposed to *M. gallisepticum* (74%) or *S. pullorum* (3.4%), which varied temporally. Although statistically independent, the relatively high coinfection rate of LPDV-infected individuals with *M. gallisepticum* (51%) or REV (10%), suggests potential immunosuppressive effects of infections. Furthermore, the cohort contributing the most to reproductive output, adult females, have the highest prevalence of infection and land type may be a mechanism explaining observed spatial variation.

The wide range in LPDV prevalence (31–65%) observed across US states and Canadian provinces may reflect both spatial and temporal variation, as sample collection spans about a decade across these studies. Our estimated LPDV prevalence in Maine's wild turkeys is similar to that in New York State (55%; Alger et al. 2017) and Ontario (65%; MacDonald et al. 2019b), but higher than other areas, including South Carolina (45%; Allison et al. 2014) and Manitoba (31%; MacDonald et al. 2019a). These data suggest that the northeastern region of North America may be a hotspot for LPDV transmission compared with southern and western regions, which is also supported by Thomas et al. (2015).

Age has been identified previously as a predictor of LPDV infection (Thomas et al. 2015; Alger et al. 2017; MacDonald et al. 2019a). High LPDV prevalence in adults could be explained

by both increasing exposure to the virus over time and chronic infections, where individuals may tolerate and harbor the virus without clearing infection. Alternatively, if LPDV infection increases the probability of mortality in juveniles, as seen with domestic turkeys (Biggs 1997; Gazit and Yaniv 1999), the removal of infected juveniles from our sample may result in a seemingly lower prevalence in this age group.

Females were more likely than males to be infected with LPDV, which agrees with previous surveys of asymptomatic wild turkeys (Alger et al. 2017; MacDonald et al. 2019a), but differs from another (Thomas et al. 2015); however Thomas et al. (2015) had a sex-biased sample, with 82% of all individuals being male. Sex variation in sociality, movement, or diet, and thus foraging behavior, as well as physiology (i.e., hormones) may influence pathogen exposure or immune response, affecting susceptibility to infection (Zuk and McKean 1996). While the potential ecological or biological mechanisms of these risk factors remain unknown, there may be population level consequences due to the greater reproductive contribution of adult compared to juvenile females (Vander Haegen et al. 1988; Roberts et al. 1995) and males.

Variation in LPDV occurs spatially and in association with landscape variables. In winter, we identified spatial variation at the regional scale as a predictor of LPDV infection in wild turkeys. Most of our samples cluster within the center of the state, however, our spring sampling omitted two regions (E and G, Figure 2.1), which happen to be among the lowest in LPDV prevalence in winter birds; therefore, future sampling should focus on broadening this spatial scope to validate these findings. We found land type as a driver of LPDV, where a decrease in agricultural land (for juveniles only) and an increase in forested land was associated with increased LPDV infection, contrasting findings from Alger et al. (2017). In northern North America, agricultural fields are important for foraging of overwintering turkeys (Vander Haegen

et al. 1989) and may provide critical nutritional resources to maintain energy put towards host immunity (Gustafsson et al. 1994). Additionally, as agricultural land becomes more abundant, turkeys may spread out over the landscape and reduce space use and movement, which can decrease contact rates and exposure to directly transmitted pathogens.

Seasonality in LPDV infection, with higher prevalence in the spring compared to winter, could be based on changes in movement, host community interactions, and reproduction as seen in other host-pathogen systems (Brown et al. 2012). Female wild turkeys have been shown to increase movement rates during pre-incubation (Chamberlain and Leopold 2000) and males demonstrate increased daily movement and distance between successive roosts during spring compared to other seasons (Holdstock et al. 2006), possibly translating to an increased rate of contact and potential exposure during spring. Furthermore, physiological changes associated with reproduction over seasons could introduce stress and subsequently increase susceptibility or exposure to viruses, as has been observed with Hendra virus in little red flying foxes (*Pteropus scapuulatus*), in which reproductive stress during spring mating was associated with increased viral prevalence (Plowright et al. 2008). Individuals may contribute more energy to reproduction, while they compete for mates, prepare nests, and lay eggs, rather than to immune defenses (Gustafsson et al. 1994).

Alternatively, seasonal differences in LPDV prevalence could be explained by how data were collected, including collection method (i.e., hunter-harvested or live-captured), sample type (bone marrow or blood), or sex-biased sampling. As bone marrow was collected from all hunter-harvested turkeys during spring, and blood from all live-captured birds during winter, we are not able to disentangle season, sample type, and collection method. It is plausible that hunters are more likely to harvest infected, and possibly weaker individuals, leading to an observed higher

infection prevalence in the spring-hunted group. In addition, spring collection targeted only males, while winter included both sexes; however, we also found that males had a significantly lower infection prevalence than females and therefore a bias in sex ratios across seasons is unlikely to explain the observed seasonal variation in prevalence. Further research using the same collection method and sample type across seasons is needed to examine the role of season in LPDV prevalence.

Annual variation in prevalence of all four pathogens in our study suggests a need for further temporal monitoring. An effect of year may be driven by a correlation with other unknown factors that vary over time, such as average winter temperature or snow depth. The difference in LPDV prevalence by season may represent an effect of year, since LPDV prevalence was higher in 2017-2018, when we also conducted spring sampling, than in 2019-2020, when sampling was limited to the winter. Consistent spatiotemporal monitoring of LPDV could shed light on pathogen dynamics and the underlying mechanisms of annual variation.

One pathogen infection may reduce host immunity to another, as seen with coinfections of REV and ALV (Dong et al. 2014, 2015), a retrovirus closely related to LPDV (Chajut et al. 1992; Allison et al. 2014). In our study, LPDV infection was not dependent upon another pathogen infection (*M. gallisepticum*, REV), or vice versa. Even so, our LPDV-REV coinfection rate of 10% is higher than asymptomatic birds reported from Manitoba (0%; MacDonald et al. 2019a) and Ontario (4% of LPDV-positive individuals; MacDonald et al. 2019b), but similar to 8% of LPDV-positive diagnostic cases presented with neoplasia (Niedringhaus et al. 2019). It is possible that the higher LPDV-REV coinfection in Maine turkeys is due to higher incidence and overlap of the viruses, driven by common factors influencing transmission dynamics (i.e., availability/prevalence of other hosts and/or vectors). Alternatively, individuals may be similarly

exposed to other parasites and environmental stressors that reduce overall immunity (Svensson et al. 1998; Kamath et al. 2014). Further study is needed on interactions between LPDV, REV and host immune response in wild turkeys to determine whether coinfections may have subclinical effects on population fitness.

Beyond the concern of pathogen transmission within wild turkey populations, especially with regards to translocation campaigns and recent population increases, there may also be a risk of spillover to poultry and other game birds, justifying consistent monitoring of these pathogens and further work to understand spillover potential. Furthermore, the increased likelihood of LPDV infection in adult females warrants further investigation into individual fitness effects that may impact population dynamics. With heterogeneity in the effect of spatial and land type risk factors dependent upon age, and a large discrepancy in prevalence between age classes, a challenge study is justified to further assess transmission mechanisms that may explain age-related variation in infection. Our findings coupled with continued monitoring can help predict spatial and temporal dynamics of LPDV in wild turkeys and inform management decisions regarding translocations and harvest regulations.

CHAPTER 3

RETROVIRAL INFECTIONS AFFECT SURVIVAL AND REPRODUCTION OF FEMALE WILD TURKEYS

Pathogens can regulate or decimate populations of free-ranging wildlife. Wild turkeys (*Meleagris gallopavo*), in particular, are a prized upland gamebird that recently experienced dramatic increases in population size and range expansion as the result of reintroduction campaigns, which may promote the transmission of directly-transmitted diseases. Factors impacting survival and reproduction have been extensively studied in wild turkeys, but few studies, if any, have examined the role of pathogen infections on demographic metrics. Lymphoproliferative disease virus (LPDV) and reticuloendotheliosis virus (REV) are avian oncogenic retroviruses that infect poultry and wild turkeys, which can result in disease and mortality, though most infected wild individuals appear asymptomatic. We investigated whether retroviral infection and coinfection influenced fitness characteristics of wild turkeys by evaluating the effects of infection on female survival rate and several reproduction metrics, including daily nest survival rate, clutch size, nest initiation timing (Julian day of first egg laid), nesting propensity (rate at which a female nested), and hatch rate (rate at which available eggs hatched). We live-captured 163 female wild turkeys throughout central Maine during three winters (January–March), from 2018–2020. We collected blood from each individual for LPDV and REV molecular diagnostics and attached either a GPS or VHF transmitter to monitor survival and nesting. The weekly survival rate for REV-infected hens was 0.973 (95% CI: 0.954–0.985), compared to 0.984 (0.975 – 0.989) for their uninfected counterparts, which translates to REV-infected individuals having nearly half the cumulative annual survival probability. Infection with LPDV was a significant predictor of clutch size ($\beta = -1.43$; 95% CI =

-2.24—0.63; $p < 0.01$), with LPDV-infected hens laying an average of 1.43 fewer eggs. We did not detect an effect of retroviral infection on nest initiation, nesting propensity, or hatch rate; nor did we find a relationship between coinfection and any measured fitness metric. These findings demonstrate that infection with REV and LPDV impacts survival and reproduction, respectively, of female wild turkeys, even in the absence of overt disease. Furthermore, this highlights the importance of considering pathogen infection when assessing factors affecting demographic metrics of free-ranging wildlife.

Introduction

Pathogens may regulate (Dobson and Hudson 1992), decimate (McCallum et al. 2009; Dadam et al. 2019), or cause no apparent harm (Kilpatrick et al. 2006) to populations of free-ranging wildlife. The health of both vulnerable (Pedersen et al. 2007) or common species (Hochachka and Dhondt 2000) can be threatened by pathogen infections. Pathogens can affect population dynamics through direct effects of infection on survival (i.e., causing mortality; Palinauskas et al. 2018), reproduction (i.e., number of chicks fledged; Pigeault et al. 2018), or both (Lachish et al. 2012). Some species, though, can harbor chronic pathogen infections and suffer no cost to survival, reproduction, or population growth, as demonstrated in Hawaiian honeycreeper (*Hemignathus virens*) experiencing chronic malarial infections (Kilpatrick et al. 2006).

While pathogen prevalence data provides insights into host infection levels and spatiotemporal distributions, these data alone do not reveal the individual- or population-level fitness consequences of infection or the level of necessity or ability to implement management strategies. For instance, 90% of keelback snakes (*Tropidonophis mairii*) were infected with haemogregarine blood parasites, but no relationship was found between infection and

measurements of host fitness (Brown et al. 2006). McCallum and Dobson (1995) highlighted the need to go beyond pathogen prevalence to identify the potential effects of infection on population vital rates, which both dictate population growth (Gotelli 2008) and individual fitness (Metcalf and Pavard 2006), in order to better understand host-pathogen dynamics and threats to biodiversity. Boadella et al. (2011) emphasized the importance of choosing specific and appropriate parameters for monitoring that accurately reflect changes in disease occurrence, recognizing prevalence rates alone may have limited value. Accounting for variability in how infection affects particular fitness metrics, while also considering potentially confounding spatiotemporal dynamics and host factors that may influence those metrics, is required before any one vital rate can be singled out as a potential index of fitness. Identifying appropriate fitness metrics and quantifying their relationships with pathogen infections is critical for modeling the and predicting the efficacy of disease management interventions; for example, simulation modeling revealed selective culling would not be an effective strategy for reducing facial tumor disease in Tasmanian devils (*Sarcophilus harrisii*; Lachish et al. 2010).

Life history tradeoffs among vital rates may be modulated by pathogen infection, and may interact with environmental factors, further complicating our ability to measure pathogen effects on host populations. For instance, when avian cholera exposure was high, common eiders (*Somateria mollissima* sp.) that devoted more energy to large clutch sizes experienced a reduction in survival, a cost that was not evident when exposure was low or absent (Descamps et al. 2009). In Soay sheep (*Ovis aries*), elevated immune response (i.e., as measured by antinuclear antibodies) was associated with increased overwinter survival and decreased fecundity, but only during parasite-induced population crash years characterized by high population density, harsh weather, and low resource availability (Coulson et al. 2001; Graham et al. 2010); in contrast,

during non-crash years, high antibody levels were associated with reduced sheep survival and increased fecundity in females. This suggests that the fitness costs of infection can fluctuate with pathogen prevalence (Descamps et al. 2009), or with the occurrence of environmental stressors that may affect immune function (Svensson et al. 1998).

Intrahost pathogen diversity, or coinfection, is another key factor contributing to the dynamic nature of host-pathogen relationships that is often overlooked (Johnson and Hoverman 2012; Cassirer et al. 2018). Coinfection, which refers to more than one distinct infectious agent simultaneously infecting a single host (Cox 2001), can result in synergistic negative effects on fitness, such as increased host mortality (Johnson and Hoverman 2012). In addition, direct or indirect (i.e., through host immunity) within-host interactions between multiple pathogen types can also lessen the effects of infection on the host; for example, mouse mortality was delayed considerably in experimental coinfection with a specific cerebral malaria species (*Plasmodium berghei*) and helminths compared with malarial infection alone (Knowles 2011). The impact of dual pathogen infection on particular fitness metrics can also appear neutral (i.e., no difference in mortality with single or multiple infections; Palinauskas et al. 2018). Furthermore, there are various underlying mechanisms that can facilitate the same observed outcome on fitness metrics (Palinauskas et al. 2018). The complexity associated with multiple infections highlights the need to consider pathogen diversity and intracommunity (within host) ecology in efforts addressing infection outcomes (Telfer et al. 2010).

Lymphoproliferative disease virus (LPDV) and reticuloendotheliosis virus (REV) are avian oncogenic retroviral (family Retroviridae) pathogens that occur at the wildlife – domestic animal interface. Lymphoproliferative disease virus was previously known to only infect and cause lymphoid tumors and mortality in domestic turkeys in Europe and Israel (Biggs et al. 1978;

Biggs 1997). Reticuloendotheliosis virus was similarly first detected in a domestic turkey (Robinson and Twiehaus 1974), and is typically associated with runting syndrome or tumor growth and immunosuppression in poultry (Fadly et al. 2008). Furthermore, REV and avian leukosis virus (ALV) coinfection has a synergistic effect on chickens, increasing mortality, immunosuppression, and tumor growth (Dong et al. 2014). Lymphoproliferative disease virus is closely related to ALV (genus Alpharetrovirus; Chajut et al. 1992), justifying concern for potential negative synergistic effects of LPDV-REV coinfection similar to that seen in REV-ALV coinfections (Dong et al. 2014).

The majority of concern and research on LPDV and REV has focused on domestic birds. However, interest in LPDV in wild avian hosts has recently spiked due to its detection in wild turkeys (*Meleagris gallopavo*) in the United States (Allison et al. 2014). Similarly, wild avifauna have gained attention as a potential source of REV infection for endangered Attwater's prairie-chickens (*Tympanuchus cupido attwateri*; Ferro et al. 2017). Wild turkeys, particularly, were the source of REV infection and caused mortality of nearly 50% of prairie chickens in a captive breeding facility (Stewart et al. 2019). Niedringhaus et al. (2019) recently issued a plea for further research to characterize the threat of LPDV and REV infection on wild turkey health. While LPDV and REV coinfections in wild turkeys have been documented (Shea et al. *in review*.; Allison et al. 2014; MacDonald et al. 2019b, 2019a), and neoplasms have been observed in wild turkeys infected with LPDV or REV submitted as diagnostic cases (Allison et al. 2014; Niedringhaus et al. 2019), it remains unknown how infections or coinfections of these pathogens affect wild turkey health, particularly in terms of fitness consequences. Furthermore, adult females, the cohort that contributes the most to reproductive output, has a disproportionately higher likelihood of LPDV infection (Shea et al. *in review*; Alger et al. 2017). Fitness metrics

vary in relative contributions to population growth (i.e., their elasticity; Caswell 2006), which can vary by age class (Blomberg et al. 2021); thus, assessing whether age variation in infection prevalence translates to age-dependent differences in pathogen-related fitness consequences is warranted.

Herein we present the first report of individual fitness effects of LPDV and REV infection and coinfection in a natural population of wild turkeys. With a relatively high reported prevalence of both pathogens in wild turkeys in Maine (REV: 16%; LPDV: 59%) and a disproportionately higher probability of infection for adult females (Shea et al. *in press, in review*), our objectives were to (1) examine the effects of LPDV and REV infection and coinfection on vital rates in female wild turkeys and (2) determine if these effects were age-dependent. Particularly, the vital rates we investigated include weekly survival rate, daily nest survival rate (DNSR), clutch size (number of eggs laid), nest initiation date (Julian day of first egg laid), nesting propensity (rate at which a female nested if she was available to do so), and hatch rate (rate at which eggs available hatched). These data will provide valuable information to evaluate the risk of retroviral infections on wild turkey survival and reproduction, as well as the long-term effects these infections on population dynamics.

Materials and Methods

Field Methods

We captured 163 live female turkeys over three winter seasons (Jan–Mar, 2018–2020) using rocket or drop nets from 29 capture sites, located mostly in central and southern Maine (Figure 3.1). For all captured birds, we recorded year of capture, body mass, and determined age and sex (Dickson 1992), where age was either adult (>1 year old) or sub-adult (born the previous nesting season, <1 year old).

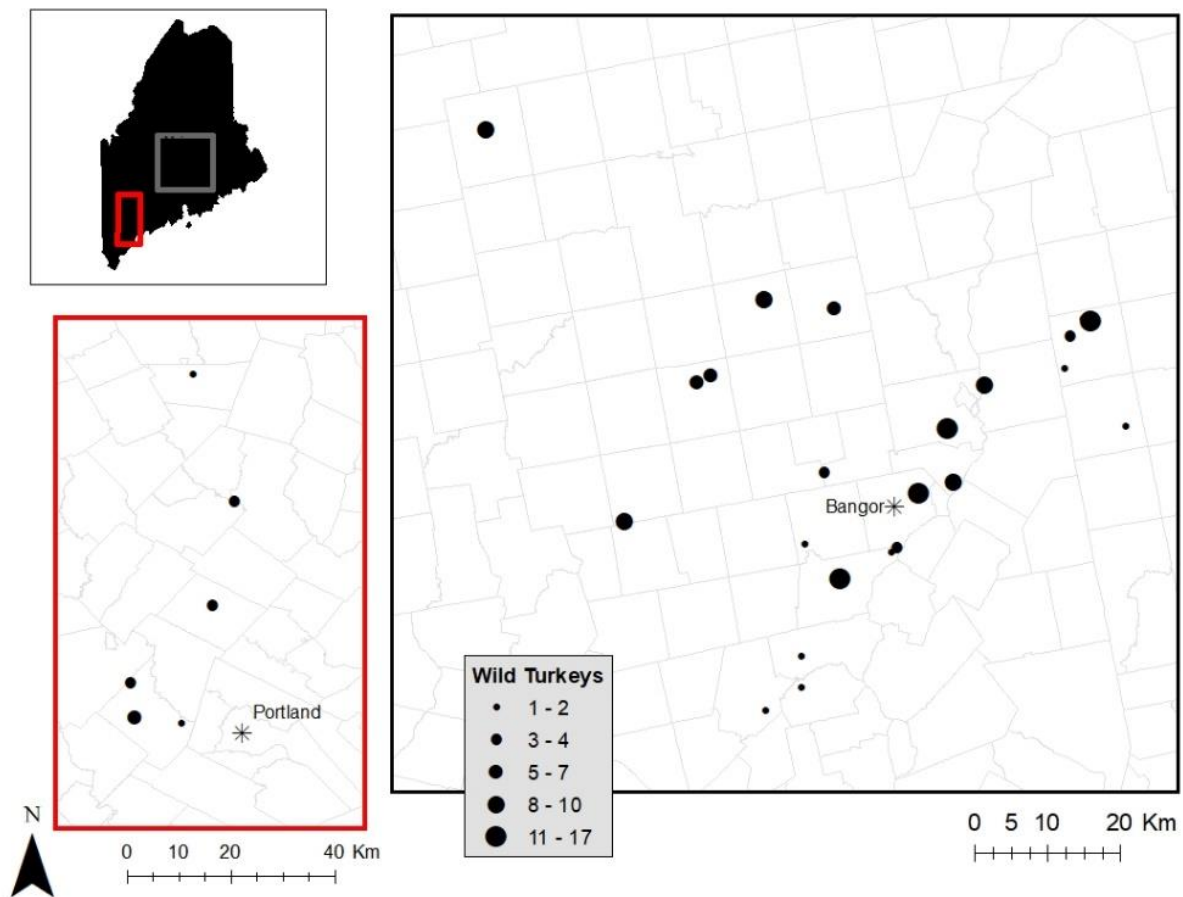


Figure 3.1. Capture site location of 163 wild turkeys fitted with GPS or VHF transmitters from 2018–2020 in Maine. Capture sites are sized by number captured.

For the purpose of analyses, a sub-adult at capture remained a sub-adult through its first nesting season, but was considered an adult beginning August 1st of the year of capture, to differentiate first-time breeders from those with past breeding experience. Each captured female was fitted with one of three transmitter models: (1) an 80g VHF backpack-style harness transmitter ($n = 91$; Advanced Telemetry Systems, Isanti, Minnesota), (2) a 90g GPS backpack-style harness transmitter with a built in VHF component ($n = 46$; Lotek Wireless Fish and Wildlife Monitoring, Newmarket, Ontario, CA), or (3) a 12g VHF necklace transmitter ($n = 26$; Advanced telemetry Systems, Isanti, Minnesota). For molecular diagnostics of LPDV and REV, whole blood was drawn from the brachial vein into an EDTA tube (1–5 mL; $n = 129$) or from a foot venipuncture into a heparin-treated capillary tube and stored in queen's lysis buffer (~1mL, $n = 34$). All capture, handling, and sampling of wild turkeys was approved by the University of Maine Institutional Animal Care and Use Committee (IACUC Protocol # A2017_11_03).

Laboratory Methods

We used a molecular approach to determine the LPDV and REV proviral infection status of all sampled individuals. From the majority of blood samples ($n = 127$), we isolated the buffy coat layer by centrifuging for 15 minutes at 2500 RPM. In some cases ($n = 36$), when blood volume was too low for buffy coat optimization or when blood was collected via capillary tubes, we used whole blood. We extracted genomic DNA from both buffy coat and whole blood using Qiagen DNeasy Blood and Tissue Kits (Qiagen, Valencia, CA), following manufacturer's instructions. For each extraction, we included a negative control and quantified DNA concentration using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) or Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA). For LPDV detection, we used previously established PCR cycling conditions (Alger et al. 2015), reagent concentrations

(Shea et al. *in press*), and primers (Allison et al. 2014) to amplify a 413 base pair region of the retroviral *gag* gene. For REV detection, we used previously established PCR cycling conditions, primers (Bohls et al. 2006), and reagent concentrations (Shea et al. *in review*) to amplify a 580 base pair region of the retroviral *pol* gene. Target region amplification was verified by electrophoresis, using a 1% agarose gel, and visualized with an Azure c150 Imaging System (Azure Biosystems, Dublin, CA). All PCR results were confirmed by either re-running PCR-negative samples or sequencing PCR-positive products to rule out false positive or negative results.

Monitoring Survival and Reproduction

We used both GPS and VHF transmitters to monitor survival of 163 individuals and 111 nests. We tracked all individuals from their respective date of capture through the week of November 14th, 2020. Individual length of time varied, but our first capture was during the week of February 3rd, 2018, so the maximum time for an individual was monitored was 146 weeks.

We used a hand-held three element directional antenna and receiver to record signals from each bird with a GPS or VHF transmitter. GPS transmitters logged locations every hour during daylight (shifted periodically) from November through July, with an additional overnight location to record roosting sites. To preserve battery life, daytime points were reduced to a morning (9am), afternoon (3pm) and roost location from August through October. Data was downloaded remotely from the transmitter using a Pin Point Commander unit with an ultrahigh frequency (UHF) connection. For GPS birds, we downloaded waypoint files weekly from the transmitters and uploaded these data to Movebank.org (Kranstauber et al. 2011) to maintain a backup and for easy conversion, viewing and analysis. The survival status (live or dead) was determined from downloaded location data, with potential mortality inferred by sequential points

at a single location. We obtained signals from VHF-marked birds approximately once a week to record location, and the survival status (live or dead) was determined based on the speed of the transmitter signal. If either a GPS- or VHF-tagged bird was suspected dead, the transmitter was approached to confirm survival status. All birds were monitored with increased frequency for two weeks following their trapping event to detect capture-related mortality, and we censored all birds that died during this time.

Female wild turkeys were monitored from April 15th to July 30th, each year of the study, for suspected nesting behavior. During this time, VHF-marked hens were monitored more frequently, and locations were collected at least twice a week via short-distance triangulation. Hens were assumed to be on a nest if found alive in the same location during two successive visits. After two weeks, the location of the hen was approached to locate and confirm nesting. If nesting, clutch size was recorded, the hen was flushed, and 3-4 eggs were selected for flotation to determine incubation stage. From the estimated incubation stage, both the initiation date of the nest (Westerskov 1950) and the hatch date were predicted. We continued to monitor the nest at least once a week with increased visits surrounding the suspected hatch date for confirmation of this estimate. Once the hen was suspected to have left the nest, we approached to determine the fate of the nest (hatched or failed) and, when applicable, record the number of unhatched eggs.

For GPS-marked hens, location data was downloaded weekly during the nesting season and point locations were viewed in Google Earth. We assumed the hen was nesting if repeated visits were made to a single location around the same time of day. Once the hen resumed regular non-nesting movements or discontinued regular daily visits, in the case of failure during the laying phase, we observed the suspected nest to verify the nest and its fate. Nest initiation was estimated from these data based on when the hen originally started to visit the site daily,

indicating the initiation of laying. Clutch size was estimated based on how many days the hen was laying (assuming one egg per day; Williams et al. 1971) prior to remaining in the same location the majority of the day, indicating the start of incubation. GPS-marked hens were not disturbed during nesting, which we were able to compare to hens that were flushed and regularly visited (VHF-marked hens) to evaluate the effect of nest monitoring on DNSR.

Encounter History

We compiled weekly status (live/dead) for each GPS- and VHF-marked wild turkey to develop a weekly encounter history, which included the week the turkey was captured, the last week it was found alive, the last week it was checked, and its final status at the end of the monitoring period. We increased frequency of monitoring during nesting season and similarly created an encounter history for DNSR.

Demographic Statistical Analyses

We evaluated the relationships between proviral infection status (REV, LPDV, coinfection), as independent variables, and several fitness metrics, including weekly survival rate, DNSR, clutch size for first and second nesting attempts, and nest initiation for first and second nesting attempts. All analyses were conducted in RStudio (Version 1.2.5019) using Program R (R Core Team 2021), and we used the AICcmodavg package (Mazerolle 2020) to employ a tiered AICc model selection approach. We first controlled for potentially confounding sources of variation in survival and reproduction by considering non-pathogen factors prior to evaluating pathogen effects. If there was support (<2.0 delta AICc) for any models containing non-pathogen variables, the variables were included in a new baseline model for the second step in AICc model selection, where we combined baseline model variables with pathogen variables (hereafter pathogen models). We evaluated both baseline and pathogen models against an

intercept-only null model. For the pathogen models, we included LPDV or REV infection status as a binary variable (0 = PCR negative, 1 = PCR positive), and a coinfection categorical variable with each of the following 4 levels: uninfected, infected with LPDV, infected with REV, infected with both LPDV and REV. We also included an age model (with an age variable added to the baseline null model) because we were interested in age-dependent variation in demographic values, particularly to inform future population models, and demographic estimates have been previously demonstrated to vary based on age in wild turkeys (Lehman et al. 2008; Pollentier et al. 2014). Lastly, age was also considered as a predictor in two independent models per pathogen variable, one that included an additive age term, and one that included an age interaction term with each pathogen variable. We included the interaction term because our previous work revealed that adults were more likely to be infected than sub-adults (Shea et al. *in review*), and variation in pathogen effects based on age may result in disproportional consequences to population growth.

We interpreted the significance of variables contained in supported models of the second AICc model selection by evaluating coefficients and their 95% confidence intervals (significance = confidence interval not overlapping zero). We did not use AICc model selection to estimate the effect of pathogen variables on nesting propensity or hatch rate and, thus, and instead used univariate logistic regression and Analysis of Variance (ANOVA), respectively, to evaluate whether pathogen infection was associated with significant differences in each of these vital rates. Level of significance was set at $\alpha < 0.05$. We used the binom package (Dorai-Raj 2014) to estimate prevalence and 95% confidence intervals of pathogens using the Wilson method (Wilson 1927).

Evaluating Pathogen Effects on Weekly Survival Rate

We modeled weekly survival rate using the nest survival model in the RMark package (Laake 2013). This approach best fit our study design because it allowed for irregular monitoring of individuals (Davis et al. 2018), which was inevitable following extended or irregular movements. We exponentiated weekly survival rate across 52 weeks to obtain an annual survival probability. For the first step of model selection, we evaluated turkey age, season (winter = Jan–Mar, spring = Apr–Jun, summer = Jul–Sep, and fall = Oct–Dec) and transmitter type (as either backpack-harness style where we combined GPS and VHF models, or necklace style) as predictors of survival, comparing these models against an intercept only null model (Table A.1). Since the model containing season better predicted survival than the null model, and Shea et al. (*in review*) also found evidence that LPDV infection varied seasonally, we hypothesized there might also be an interaction effect between season and infection status on survival. Therefore, for the second AICc model selection, in addition to comparing the age models specified above (interaction and additive term with each pathogen variable), we also included three additional season models, with an interaction term between season and each of the pathogen variables.

Evaluating Pathogen Effects on Reproduction

We modeled DNSR using the nest survival model in the RMark package (Laake 2013), after first censoring any nests with unknown fate. The variables included in the initial model selection step were nest age (days), turkey age (at nesting), nest attempt (first or second nest attempt within the same nesting year), Julian day of nest initiation, and nest year. We also evaluated transmitter type since, when gathering nesting data, transmitter types (GPS vs. VHF) were expected to result in different levels of disturbance to hens. Lastly, we calculated overall nest success, defined as percentage of initiated nests that survived to hatching.

We used AICc model selection on linear models to determine the relationship between pathogen infection and coinfection on clutch size (number of eggs laid) and nest initiation (Julian day of first egg laid). Clutch size reportedly varies based on nest attempt (Roberts et al. 1995); thus, we conducted preliminary linear regression analyses and confirmed that nest attempt was a significant predictor of clutch size in our study. Nest initiation inherently varies based on nest attempt, as second nesting attempts chronologically follow first nest attempts. Therefore, we subset our data to analyze the first and second nest attempts (within the same nesting year) separately for clutch size and nest initiation (third nest attempt was excluded from the analyses due to a sample size of one). The non-pathogen variables included in the initial AICc model selection for both clutch size and nest initiation were turkey age (at nesting), year (of nesting) and body weight (at capture). For clutch size, we also included two models containing either Julian day of nest initiation or the Julian day quadratic term.

We examined the relationship between pathogen infection status and nesting propensity, defined as the rate at which a female nested if she was available to do so. We used univariate logistic regression to determine if hen age, coinfection status, or LPDV or REV infection affected nesting propensity (0 = did not nest, 1 = nested). To determine nesting propensity for first, second, and third nests, we excluded individuals with VHF transmitters due to the higher potential for missed nests. We included any hen that was alive (available to nest) on the average Julian day of nest initiation specifically for each year and nesting attempt. For second and third nests, individuals were considered available to nest only if they had failed their previous attempt and were alive on the estimated average Julian day of nest initiation for that attempt in a given year. For example, in order to nest a third time, the hen had to remain alive until the average

Julian day of nest initiation for the third nest attempt of that year, but had failed the previous two nest attempts.

Lastly, we assessed the effect of pathogen infection on hatch rate of eggs in successful nests for both VHF- and GPS-marked birds. Hatch rate was defined as the proportion of eggs that hatched relative to the total number of eggs available to hatch (clutch size). We conducted a one-way ANOVA to determine if there was a significant difference in mean hatch rate based on age, LPDV, REV, or coinfection status.

Results

Evaluating Pathogen Effects on Weekly Survival Rate

We analyzed survival data of 163 wild turkey females. Pathogen prevalence and distribution of both single and dual infections are reported in Table 3.1. All infected individuals were outwardly asymptomatic, except for one LPDV/REV positive turkey with facial lesions that died 7 weeks post capture. The top performing models in the second model selection included the additive terms of season, REV, and hen age (Table A.2). Transmitter type was not a predictor of weekly survival rate, indicating transmitter placement (back or neck) or weight difference (~73g) did not impact survival. Only REV infection was statistically significant ($\beta = -0.510$; 95% CI = $-0.976 - -0.043$). When averaged across season and hen age variables, an REV infected individual had a weekly survival rate of 0.973 (95% CI: 0.954 – 0.985), compared to a rate of 0.984 (95% CI: 0.975 – 0.989) for their uninfected counterparts (Figure 3.2); this translates to an REV-infected individual having nearly half the cumulative annual survival probability of an uninfected individual (0.245 vs 0.427; 95% CI: 0.086–0.445 vs 0.268–0.574 Figure 3.2). Infection with LPDV or coinfection status did not affect survival.

Table 3.1. Pathogen prevalence and distribution in 163 wild turkey females for weekly survival rate assessment.

Pathogen	# Infected	Prevalence	95% CI
LPDV	117	71.8%	64.4–78.1%
REV	38	23.3%	17.5–30.4%
LPDV only	89	54.6%	46.9–62.1%
REV only	10	6.1%	3.4–10.9%
Both LPDV and REV	28	17.2%	12.2–23.7%
Neither LPDV or REV	36	22.1%	16.4–29.1%

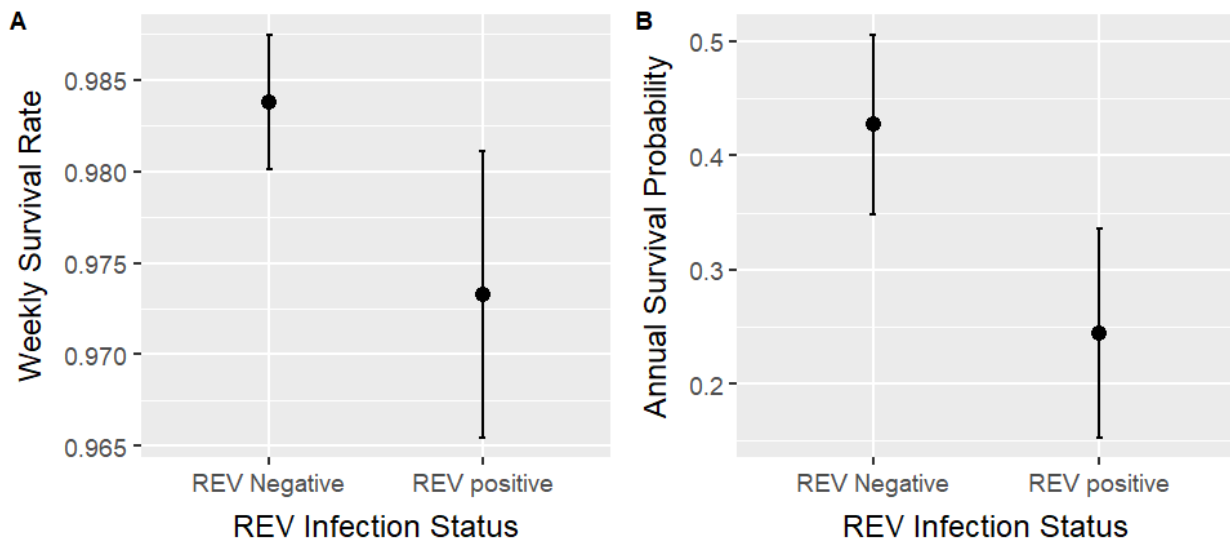


Figure 3.2. (A) Weekly survival rate and (B) cumulative annual survival probability based on REV infection status for 163 female wild turkeys captured and monitored over three years (2018–2020) in Maine. Estimates (with standard error bars) were derived from top performing models of weekly survival rate using AICc model selection.

Evaluating Pathogen Effects on Reproduction

Overall nest success was 33.3% for 111 nests, with 30.2% of first nests ($n = 96$), 50% of second nests ($n = 14$), and the single third nest (100%; $n = 1$) being successful. Location, and distribution of pathogen infection status and nest fate are shown in Figure 3.3. Pathogen prevalence and distribution of hens are reported in Table 3.2. The initial model selection indicated nest age as a predictor of DNSR (Table A.3), which was then included in all models during the second stage of model selection. For the second stage of model selection, models containing REV, LPDV, turkey age, nest age, and the interaction of LPDV and turkey age were supported (Table A.4), and we subsequently evaluated coefficients and confidence intervals of a model containing all supported variables, but only nest age was a significant predictor of DNSR, which was inversely proportional to DNSR and ($\beta = -0.060$; 95% CI = $-0.085 - -0.035$).

We evaluated whether pathogen infection affected clutch size of 107 wild turkey nests (average clutch size by hen age and nesting attempt are included in Table A.5). For the first nest attempt, the variables contained in the top-performing models of the initial non-pathogen model selection (<2 delta AICc), were nest initiation and nest initiation squared (Table A.6). In addition to nest initiation variables, the top-performing models in the pathogen model selection contained LPDV and turkey age as additive terms (Table A.7). LPDV was a significant predictor of clutch size of first nest attempt ($\beta = -1.43$; 95% CI = $-2.24 - -0.63$), with LPDV-infected hens laying an average of 1.43 (± 0.41) fewer eggs than their uninfected counterparts (Figure 3.4). Turkey age and Julian day of nest initiation did not have a significant effect on clutch size. For the second nest attempt, age was contained in a top supported model in the first AICc model selection (Table A.8), and models with pathogen variables were not supported in the second model selection (Table A.9). While age was contained in a top-performing model, the effect of age on

clutch size was not significant, potentially due to overall small sample size of second nests and uneven distribution of nests between sub-adults ($n = 2$) and adults ($n = 12$).

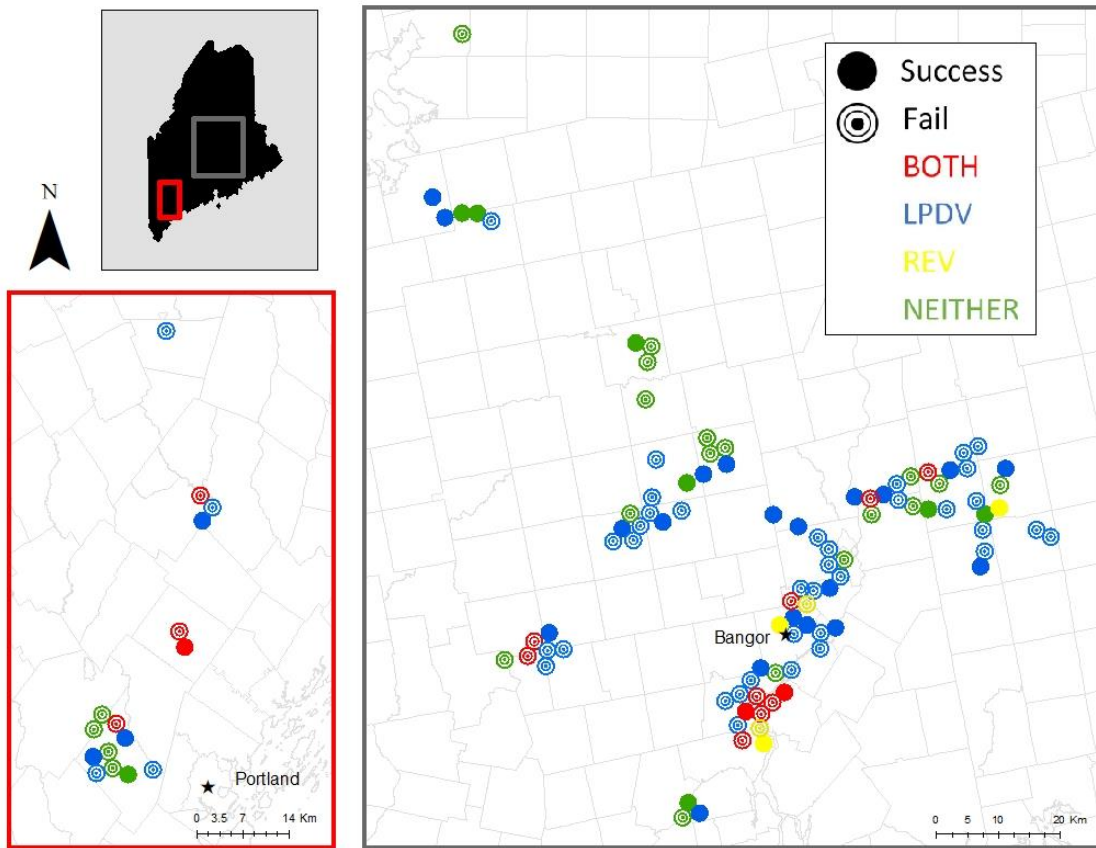


Figure 3.3. Nest locations for all nesting attempts across all three years (2018–2020) for female wild turkeys live-captured in Maine. Nest sites are colored based on the infection status of the hen (LPDV only, REV only, both, or neither) and shaped according to nest fate (success or failure).

Table 3.2. Pathogen distribution in 111 wild turkey nests based on hen infection status for daily nest survival rate assessment.

Pathogen	# Infected	Prevalence	95% CI
LPDV	77	69.4%	60.3–77.2%
REV	20	18.0%	12.0–26.2%
LPDV only	62	55.9%	46.6–64.7%
REV Only	5	4.5%	1.9–10.1%
Both LPDV and REV	15	13.5%	8.4–21.1%
Neither LPDV or REV	29	26.1%	18.9–35.0%

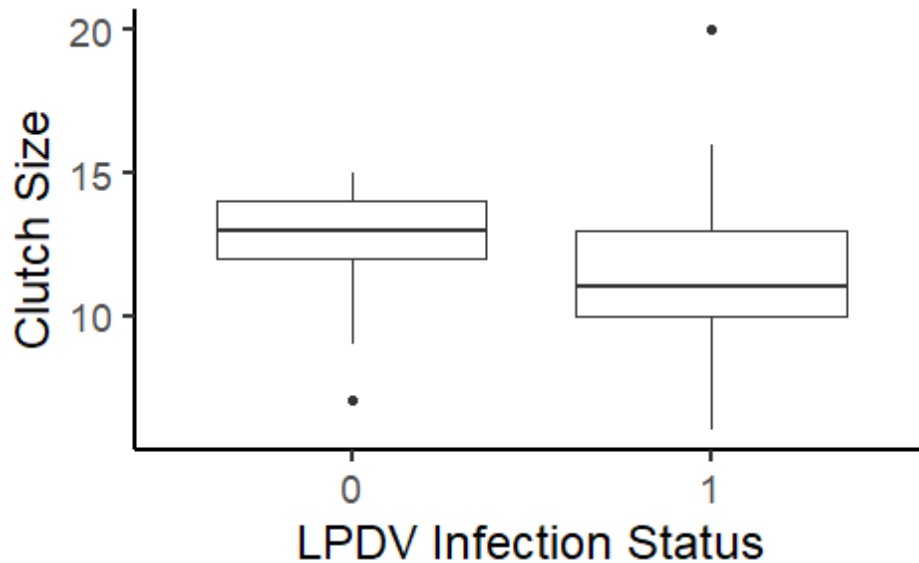


Figure 3.4. Boxplot displaying clutch size of 92 first nest attempts based on LPDV infection status over three nesting seasons (2018–2020). Estimates were derived from top performing models using AICc model selection.

We investigated whether pathogen infection or coinfection predicted nest initiation for 117 wild turkey nests (average nest initiation overall and by year, age, and attempt are in Table A.10). For the first nest attempt, the model containing nest year performed better than the null model in the first AICc model selection (Table A.11), and models including nest year, turkey age, LPDV and REV were supported in the second model selection (Table A.12). However, only nest year was found to be a significant predictor of nest initiation; nests in year 2019 were on average 8 days later than nests in 2018 ($\beta = 8.19$; 95% CI = 0.45 – 15.94). For the second nest attempt, only turkey age was included in the top-performing model of the initial model selection (Table A.13), and no additional models were supported in the second AICc model selection (Table A.14). For the second nest attempt, sub-adults have an average nest initiation about 17 days earlier than adults ($\beta = -17.07$; 95% CI = -31.80 – -2.33), though this may be at least partly attributed to an uneven sample size of only 2 sub-adults compared to 15 adults. In conclusion, pathogen infections were not found to affect nest initiation in wild turkeys.

We used data from 33 GPS-backpacked hens to estimate nesting propensity and determine if there was variation based on retroviral infection status. Nesting propensity was estimated to be 0.80 (33/41) for first nests, 0.22 (5/23) for second nests, and 0.33 for third nests (1/3). The majority of individuals available to nest in a given year were from individuals captured during the winter immediately preceding the nesting season, though 2 individuals from 2018 were available to nest in 2019 and 6 individuals in 2019 were available to nest in 2020. We did not find a significant effect of age, LPDV, REV, or coinfection status on nesting propensity for first or second nesting attempts ($p > 0.4$).

There were 40 unhatched eggs out of a total of 419 eggs available to hatch (overall hatch rate of 85.8%) in 36 successful nests (2 sub-adults, 34 adults). We did not detect a significant difference in hatch rate based on age, LPDV, REV, or coinfection status ($p > 0.2$).

Discussion

Our study provides evidence to suggest retroviral (REV and LPDV) infections negatively affect female wild turkey fitness metrics. We found that REV infection reduces annual survival by nearly half, and LPDV infection adversely influences reproduction, where an infected hen experiences a clutch size reduction of ~ 1.4 eggs. Asymptomatic pathogen infection was more common than outward disease; only one symptomatic individual was detected, which died within 7 weeks post-capture. Coinfection status did not seem to affect survival or reproduction. Overall, these data uncover the implications of asymptomatic retroviral infection on wild turkey populations and will be valuable for parameterizing models that evaluate the impacts of pathogen infection on population dynamics.

Previous studies have evaluated the effects of predation, poaching, vehicle collision (Kurzejeski et al. 1987), snow depth, food availability (Kane et al. 2007), season (Palmer et al. 1993), and dispersal distance and home range size (Hubbard et al. 1999) on the survival of female wild turkeys, but these assessments rarely include pathogen infection (Palmer et al. 1993). Considering how pathogen infections affect wild turkey survival is important given their status as a gamebird, and because infection may be enhanced and spread by reintroductions and subsequent population growth. Translocated individuals may be more susceptible to pathogen risk due to reduced genetic variation and naïve immune systems (Cunningham 1996). Additionally, harvesting combined with pathogen-mediated fitness effects may reduce population productivity (Choisy and Rohani 2006). Likewise, harvest may enhance pathogen

transmission, increasing infection prevalence and the occurrence of clinical disease (Choisy and Rohani 2006), which can further complicate the known effects of harvest on population growth and age structure (Vangilder and Kurzejeski 1995). Furthermore, translocation, density increase, and proximity to closely related wild and domestic avian species could make them more prone to infection and to facilitate spillover events (Arneberg et al. 1998; Woodford 2009), justifying incorporation of pathogen surveillance into management strategies.

We identified a significant effect of REV infection on hen survival, where infection with REV reduces the annual survival probability by approximately half (0.245 vs 0.427). With almost a quarter of wild turkey hens infected with REV, this may translate to profound effects on population demography. There are several mechanisms by which REV infection can directly impact survival, and thus fitness, in wild turkeys. In particular, REV infection and subsequent clinical disease, including tumor growth and runting syndrome (Fadly et al. 2008), can cause direct mortality in chicken flocks, with mortality reaching up to 16% (Okoye et al. 1993). In wild turkeys, REV is associated with emaciation, poor nutritional condition, and neoplasms in the skin, liver, and spleen (Niedringhaus et al. 2019). Infection with REV caused the death of nearly 50% of an Attwater's prairie chicken flock at a captive breeding facility, in which wild turkeys were implicated as the source of infection (Stewart et al. 2019). The turkeys in this study were primarily outwardly asymptomatic at capture, which suggests that direct effects of REV on mortality are either unlikely or subclinical.

This effect of REV on survival may alternatively be indirect, via interaction with other known causes of host mortality. For instance, avifauna infected with malarial parasites incur an increased risk of predation compared with uninfected individuals (Møller and Nielsen 2007). Home range size has been associated with mortality risk for wild turkey hens (Hubbard et al.

1999), such that greater movements enable increased habitat sampling and refined selection that results in increased survival; thus, it is possible that REV infection limits female habitat selection behaviors, thereby reducing home range size and increasing the subsequent mortality risk. Altered behavior has been demonstrated by house finches (*Haemorhous mexicanus*) infected with *Mycoplasma gallisepticum*, which were less mobile and more likely to be feeding alone than uninfected individuals (Dhondt et al. 2005; Hotchkiss et al. 2005). Infection may also increase susceptibility to human harvest through effects on behavior or reactionary measures, which has been observed in other gamebirds (Jackson 1969). Furthermore, vehicle collisions are also a significant cause of wild turkey mortality (Kurzejeski et al. 1987) and links have been found between pathogen infection and increased vehicle collisions in other host-pathogen systems (Schwartz et al. 2020).

Additionally, REV is immunosuppressive in chickens (Fadly et al. 2008), facilitating subsequent infection by other pathogens that may have not otherwise been equipped to surpass host immune defenses, potentially affecting host survival. Specifically, REV infection has been shown to inhibit or reduce cytotoxic T cell proliferation, decreasing the host's ability to destroy tumor cells (Rup et al. 1979; Walker et al. 1983), though this suppression appears to be transient and may require continued viral replication (Rup et al. 1979). In addition, mortality, immunosuppression, and growth retardation caused by REV are exacerbated upon coinfection with another avian oncogenic retrovirus, ALV (Dong et al. 2014), a retrovirus closely related to LPDV (Chajut et al. 1992; Allison et al. 2014). While we did not see an impact of coinfection status on survival there may be other pathogens not considered here that interact with REV in our wild turkey population.

We are not able to discern whether the reduced survival rate of REV-infected wild turkeys is representative of direct mortality, or if REV infection facilitates other stressors (such as environmental or additional parasites) that were ultimately the cause of mortality. While both direct and indirect factors are apparent in poultry, parsing the underlying mechanism of reduced survival is a common roadblock in research on wild populations (Burthe et al. 2008; Beldomenico et al. 2009). Little prior knowledge exists on the individual fitness effects of REV infection in free-ranging avifauna, and we provide the first association with deleterious impacts on survival, though further attention is encouraged to discern underlying mechanisms.

Beyond impacts to morbidity and mortality, pathogens can influence host fitness by affecting reproductive success. This can be accomplished by reducing fecundity, compromising the ability for parents to care for young, or vertical transmission of the pathogen to offspring, leading to a reduction in offspring survival (Feore et al. 1997; Lachish et al. 2012; Markos and Abdela 2016). Infection with REV did not impact any measured reproductive metric in this study, but LPDV negatively modified clutch size, which can translate into dampened fecundity and potentially affect individual fitness. Factors impacting fecundity, have been targeted as a priority for improving population growth in wild turkeys, since recruitment tends to be highly variable across populations (Roberts and Porter 1998; Pollentier et al. 2014). Additionally, LPDV is an oncogenic retrovirus that can directly influence hen condition. Allison et al. (2014) found LPDV was the likely cause of mortality in 10% of LPDV-infected wild turkeys submitted to diagnostic labs, and Niedringhaus et al. (2019) associated emaciation and neoplasms in the liver, spleen, and skin with LPDV infection. It has also been suggested that LPDV is immunosuppressive in wild turkeys based on pathogen coinfection rates (Shea et al. *in review*)

and in chickens (Payne 1998). Therefore, engaging in reproduction may require tradeoffs with immunocompetence (Svensson et al. 1998).

Egg production is energetically costly and can be vulnerable to effects of pathogen infection on hen condition. We estimated a reduction of ~1.4 eggs per LPDV-infected hen, which suggests there may be an energetic cost of immune response to infection, leading to decreased nutrients available to support egg production, to physically carry eggs, and/or to endure the laying period. Experimental induction of the immune response in house sparrows (*Passer domesticus*) was linked with an increase in energy expenditure comparable to that required to produce a single egg (Martin et al. 2002). As a precocial species, wild turkeys incorporate a large amount of energy into the yolk (Carey et al. 1980), and require specific nutrients for egg development (Perrins 1996). Once the egg is developed, egg laying is a dangerous endeavor for birds, presumably due to predation threats associated with returning to the same location each day, as well as due to the added weight from enlarged reproductive organs and an egg in the oviduct (Perrins 1996). Vangilder et al. (1987) found that only 80% of nests initiated reached incubation. In previous studies, female wild turkeys took approximately two weeks to lay a clutch (Healy 1992) and average clutch size was reportedly 11.7 for first nests across several studies (Zammuto 1986), which is strikingly similar to our average of 11.8. The number of eggs laid is also positively correlated with the duration of the laying period (wild turkeys typically lay one egg per day; Williams et al. 1971) and, therefore, larger clutch sizes not only deplete nutrient sources, but may also increase predation risk during breeding. We found that nest age was also a significant predictor of DNSR, possibly due to increased predation during later stages of nesting, though we did not identify cause of nest failure. In common eiders (*Somateria mollissima*), larger clutch size is correlated with reduced survival or breeding

probability the following year (Descamps et al. 2009), suggesting a trade-off in clutch size and future reproductive output and survival. Therefore, it is possible that wild turkeys burdened with LPDV must compensate with a reduction in clutch size to ensure survival and the prospect of future breeding (as an iteroparous species).

Wild turkey population growth models are highly sensitive to poult survival (Pollentier et al. 2014), which we were not able to address in the current study. Further research considering poult survival would be critical for determining whether the effect of infection on clutch size translates to an ultimate reduction in fecundity, or if a diminished clutch size results in increased poult survival due to curtailed requirements of parental care. For instance, the effects of Paridae pox infection on reproduction in great tits (*Parus major*) manifests at the later stage of breeding via diminished parental care post-hatching (fledge young and rear to independence; Lachish et al. 2012). Poult survival could be directly affected by pathogen infection through transmission from parent to offspring (Haider et al. 2014). More research into post-hatching effects of infection on poult survival, specifically pertaining to parental care and vertical transmission, are warranted to be able to determine the fitness cost of LPDV infection on reproduction.

Age-structured differences in survival and reproduction play an important role in in population growth dynamics (Gotelli 2008). Adults and females are more likely than sub-adults and males to be infected with LPDV (Shea et al. *in review.*; Alger et al. 2017) and seropositivity rates of REV also reportedly increased with age in chickens (Yang et al. 2017), highlighting age as a risk factor of infection. Therefore, we hypothesized that effects of LPDV and REV on individual demographic metrics may depend on age class, but we found no evidence for this. However, the higher prevalence of LPDV in adults may affect population-level dynamics, for instance, due to the dampening effect of LPDV on reproductive output in the cohort that

contributes the most to reproduction (Vander Haegen et al. 1988; Roberts et al. 1995). A population growth analysis is warranted to determine the role of LPDV infection on long-term population dynamics.

Coinfection can have varied effects on host fitness due to microbial infracommunity ecology and host immune response to multiple pathogens. We hypothesized that coinfection with LPDV and REV would intensify negative effects on demographic metrics through immune system suppression (Pedersen and Fenton 2007). Although we found approximately 13-17% turkeys were co-infected, coinfection did not decrease survival or any reproductive metric relative to single pathogen or no infection. Retroviruses are chronically integrated into the host genome, and, thus, are characterized by periods of both inactivity and active replication (Cloyd 1996; Justice IV and Beemon 2013; Rouzine et al. 2015), so it is possible that observable pathogen effects and interaction between coinfecting retroviruses are transient, whereby they do not share concurrent periods of activity. Alternatively, it is plausible that direct (resource) or apparent (immune-mediated) competition (Cressler et al. 2016) is occurring that may reduce the active period of either virus, thereby reducing possible effects on the immune system and fitness without altering the probability of proviral detection. It is also possible that additional microbes not considered in our study may be influencing infracommunity dynamics through within-host processes such as competition or synergism. Interactions among coinfecting parasites and their hosts are complex; in another host-pathogen system, two species of trematodes were found to cryptically reduce the within-host persistence of one another, but still maintain consistently positive correlations of coinfections; therefore, their coinfection rate may better be explained by among-host processes such as exposure and transmission (Johnson and Buller 2011). Expanding the number of pathogens surveyed or performing mechanistic experiments to understand the

within-host microbial interactions coupled with gaining a better understanding of among-host exposure and transmission of REV and LPDV could provide better context for deciphering coinfection dynamics.

Occurrence of overt disease first comes to mind when considering individual effects of pathogen infection. This is because many pathogens have profound visible fitness effects, such as Tasmanian devil facial tumor disease or sarcoptic mange (Hawkins et al. 2006; Scott et al. 2020). Although REV and LPDV infections in wild turkeys tend to be outwardly asymptomatic (Thomas et al. 2015), these pathogens may influence population dynamics given their widespread distribution and moderate to high infection prevalence (Shea et al. *in review.*; Alger et al. 2017; MacDonald et al. 2019b), combined with the subclinical effects on fitness observed in this study. It is also possible that individuals are symptomatic upon initial infection, which did not coincide with capture, and subsequently experience long-term fitness effects from chronic infection via retroviral integration into the host genome. Alternatively, individuals may be symptomatic after capture when the retrovirus enters an active state, perhaps when the virus has reached cells in a target-rich environment (Rouzine et al. 2015). The occurrence, level, reasons for, or consequences of active and inactive periods of REV and LPDV infection have not, to our knowledge, been addressed in wild turkeys and deserves further attention to accurately assess infection dynamics.

Furthermore, we sampled wild turkeys in winter, and assumed their LPDV and REV proviral DNA infection status remained constant across the entirety of the study, though this may not always reflect active infection as described above (Cloyd 1996; Justice IV and Beemon 2013). It is likely that proviral infection status remained constant for those infected at capture due to viral insertion into the host genome, however it may be less realistic for those uninfected

at capture because they could become infected throughout the course of the study. In the case of the latter, the strength of the negative relationships between infection and fitness metrics observed in our study are likely conservative.

In conclusion, apparently asymptomatic retroviral infections resulted in individual fitness effects that could ultimately affect wild turkey population dynamics. Little research on wild turkey survival and reproduction has included host-pathogen dynamics, and our study demonstrates that this is a key component necessary for understanding population dynamics. Future research should prioritize applying these findings to population growth models to determine population-level impacts of retroviral infections, considering pathogen infection and the age-structured relative importance of fitness metrics. Furthermore, evaluating indicators of host immunocompetence and immune response to LPDV and REV could provide insight into underlying mechanisms resulting in reduced fitness.

CHAPTER 4

GENETIC DIVERSITY AND STRUCTURE OF LYMPHOPROLIFERATIVE DISEASE

VIRUS IN A RECENTLY REINTRODUCED WILD TURKEY POPULATION

Wild turkey (*Meleagris gallopavo*) reintroduction campaigns have led to widespread increases in population density and rapid range expansion. This rapid population growth and expansion has put the species into proximity with closely related wild and domestic birds, making them more prone to infection as well as potential sources of disease spillover.

Lymphoproliferative disease virus (LPDV) is an avian oncogenic alpharetrovirus that occurs at the wild – domestic interface as it infects wild turkeys across North America and has been found to also infect domestic turkeys. While previous work has found LPDV to be genetically diverse and widespread in wild turkeys of North America, little is known about strain diversity or transmission dynamics at local scales. Therefore, we sought to assess the genetic diversity, spatial structure, and evolutionary history of LPDV strains infecting wild turkeys in Maine. We collected tissues from 627 live-captured and 72 hunter-harvested wild turkeys over four years (2017–2020) in Maine. We PCR-amplified a 413 base pair region of the *gag* region of LPDV. Positive PCR-products from 409 individuals were sequenced and revealed high haplotype ($H_d = 0.982$) and nucleotide diversity ($\pi = 0.015$), with 229 unique LPDV strains found in Maine's wild turkey population. The majority of individual turkeys possessed unique strains of the virus, with only 27% of strains shared by two or more birds, further illustrating high genetic diversity across our sample. We estimated phylogenetic relationships among LPDV strains using Bayesian inference, which revealed a lack of discernable geographic structure in the genetic data. However, a Mantel's test revealed a significant, albeit weak relationship between geographic and genetic distance ($R^2 = 0.004$, $p = 0.033$). Together, our data suggest rapid expansion of the virus

and more recent genetic divergence, as recent wild turkey populations have spread and become established throughout the state. Our results on a localized state-scale were similar to that previously found at the larger continental scale in the eastern US. These baseline data are useful as contributions to future monitoring and genetic diversity assessments, which will require higher resolution genetic data to evaluate LPDV transmission dynamics on both finer and broader spatial scales.

Introduction

While the majority of pathogen transmission and evolution occurs in free-ranging wildlife, knowledge on the subject is limited compared to that of domestic species (Caron et al. 2012). Host-pathogen dynamics and evolution in the wild can have profound effects on wild and domestic animal health, human health, and biodiversity (Daszak et al. 2000). For instance, zoonoses comprise 60.3% of human emerging infectious diseases (EID) with 71.8% deriving from wild animal sources (Jones et al. 2008) and a disproportionate amount of these diseases are attributed to RNA viruses (Holmes 2009). Additionally, spillover events from wild to domestic animals could be catastrophic to poultry operations and result in massive economic losses. In 2014, an epidemic of highly pathogenic avian influenza virus that resulted from a spillover event from wild birds devastated the US poultry industry with the depopulation of almost 50 million chickens and turkeys, costing taxpayers nearly \$1 billion (USDA 2016). Pathogen genetic data have been used to evaluate transmission within and between host species, pathogen demographics, and rates of spread. Understanding the potential consequences of pathogens occurring among species starts with researching the pathogen dynamics in its natural host and ecosystem.

Lymphoproliferative disease virus (LPDV) is an avian oncogenic RNA alpharetrovirus that has been found to infect both wild and domestic turkeys (Biggs 1997; Allison et al. 2014). In North America, the virus was recently detected and found to be widespread (26 – 83% by state/province) in wild turkey populations (Allison et al. 2014; Thomas et al. 2015; MacDonald et al. 2019a, 2019b), but has not been found to infect domestic turkeys (MacDonald et al. 2019b). LPDV infections were documented in domestic turkeys in Europe and Israel (Biggs 1997), and were associated with overt disease, high flock mortality, and horizontal transmission (Biggs et al. 1978; McDougall et al. 1978; Gazit and Yaniv 1999). In wild turkeys, however, overt disease is uncommon, though it has been documented in wild turkeys submitted to diagnostic labs, where it has also reportedly resulted in mortality (Allison et al. 2014; Thomas et al. 2015; Niedringhaus et al. 2019). Therefore, the potential for domestic turkeys to act as a disease reservoir and source of infection to wild turkeys remains a real concern. Despite this, little is understood about LPDV transmission or evolution in natural wild turkey populations.

Phylogenetic analyses of pathogen genetic data allow the reconstruction of evolutionary relationships among variants, or strains, which can provide information about transmission pathways among individual hosts (Bouwstra et al. 2015). Evaluating evolutionary relationships among intraspecific isolates can provide information regarding pathogen genetic diversity and structure within or among host populations, as demonstrated through a phylogenetic analysis of *Mycobacterium tuberculosis* (Filliol et al. 2006). In addition, examining how strains have evolved from their common ancestor can provide insights into potential mechanisms promoting pathogen evolution, for example through the identification of strains undergoing rapid divergence, which in turn may affect infection dynamics (Kerr et al. 2019). The topology of pathogen phylogenies alone has been used to resolve patterns in transmission underlying an

outbreak (Colijn and Gardy 2014). Furthermore, investigating pathogen phylogenies across space and time, in association with known host and pathogen characteristics, can provide insight into the drivers and pathways of pathogen transmission and evolution, data which is needed to better understand host-pathogen relationships, identify transmission hotspots, and assess spillover potential.

Genetic diversity can provide insight into how long a pathogen has been coevolving with its host. High genetic diversity is often associated with high effective population sizes (Hague and Routman 2016), and pathogen variation enables adaptation for persistence within hosts (Anderson and May 1982), as well as to spillover to new host species. High pathogen genetic variation is expected in reservoir hosts, as they carry more diverse strains in contrast with spillover hosts that experience a transmission bottleneck, where few strains are passed on through the spillover process (Pybus and Rambaut 2009). This is demonstrated by high diversity of *Mycoplasma ovipneumonia* in domestic sheep, suggesting they are a reservoir host, in comparison to the few strains circulating in bighorn sheep or mountain goats, believed to be the spillover hosts (Kamath et al. 2019). It remains unknown whether LPDV is an emerging pathogen in wild turkeys or one that has remained undetected due to lack of surveillance. Quantifying genetic diversity can address this gap in knowledge by illuminating host-pathogen history, which may provide insights into whether LPDV is endemic or the result of recent spillover into wild turkey populations.

Incorporating geographic information into phylogenetic analysis using strain sequence data could elucidate infection sources, outbreak dynamics, and patterns in transmission pathways and spatial spread among hosts. In particular, a single bacterial strain found across multiple populations of a single host species in a localized geographic space could be the result of a single

spillover event, amplified through individual host contacts and subsequent geographic spread (Cassirer et al. 2018). Alternatively, multiple genetically distinct strains occurring within a single population in the same geographic region could portray multiple pathogen introductions into the population (Cassirer et al. 2018). Furthermore, high genetic diversity and high rates of accumulating nucleotide substitutions of foot-and-mouth-disease virus during an outbreak in the United Kingdom allowed for the reconstruction of particular transmission pathways between individual livestock (Cottam et al. 2006). An outbreak of bluetongue virus in Europe in 2006 was traced to Sub-Saharan Africa as the source location through a comparison of strain types from the outbreak to other geographic locations (Maan et al. 2008). Furthermore, Identifying LPDV sources and transmission pathways is critical for determining infection hotspots and the potential risks of spillover.

Pathogens typically evolve more rapidly than their hosts due to faster mutation rate and shorter generation time (Rannala and Michalakis 2003; Whiteman and Parker 2005). This allows the use of pathogen genetic data across space and time to infer host population structure and recent demographics over shorter time periods (Biek et al. 2006), which can be valuable for detecting host population responses on relevant time scales for managing threatened or endangered species, such as responses to anthropogenic perturbations. Cougar population growth facilitated by legal protections (following drastic declines) was inferred from the genetic structure and prevalence data of feline immunodeficiency virus (FIV) in the cougar population (Logan and Sweanor 2001; Biek et al. 2006). The ability for viral genetic data to illuminate host population structure and demographic history is particularly relevant because wild turkeys, too, experienced a massive contraction in their geographic range and population size across North America (Aldrich 1967; Kennamer et al. 1992). Successful reintroduction efforts restored the

population beyond its original range, including in Maine with populations growing from 0 to 60,000 in just 40 years (Allen 2000; Sullivan 2017). It is possible that evidence of host population growth, dispersal, or connectivity are discernable using viral strain diversity data.

Previous phylogenetic analyses of LPDV proviral sequences have consistently revealed two primary clades: the first clade is comprised of the Israeli prototype strain, a molecular clone isolated from a domestic turkey (Sarid et al. 1994), clustered with four viruses from South Carolina and the second clade is comprised of all other viruses analyzed from North American states and provinces, with Colorado as the western-most point (Allison et al. 2014; Thomas et al. 2015; MacDonald et al. 2019a, 2019b). Phylogenetic analysis by both Allison et al. (2014) and Thomas et al. (2015) included sequences from 17–18 states, but contained relatively small sample sizes within each state (except for New York in the latter publication which contained 132 samples), with the few sequences obtained from wild turkeys in Maine also grouping with most North American strains. While these studies were able to discern some degree of clustering by state on a continental scale, genetic strain diversity and structure at finer scales has not previously been investigated.

Much remains unknown regarding LPDV strain diversity and transmission in wild turkeys at finer spatial scales. Consistent monitoring of strain diversity would allow for early detection of newly evolving strains that may be more virulent or support increased transmission potential (Lee et al. 2012). To address this gap in knowledge, we used LPDV proviral sequence data to assess the genetic diversity, spatial structure, and phylogenetic relationships of LPDV strains infecting wild turkeys in Maine. These data illuminate the evolutionary history and transmission of LPDV in wild turkey populations and provide insights into the role reintroductions play in shaping pathogen infection dynamics.

Methods

Field Methods

We collected biological samples from 699 hunter-harvested and live-captured wild turkeys for molecular diagnostics of LPDV, located across 13 (out of 29) Wildlife Management Districts (WMDs) in Maine (Figure 4.1).

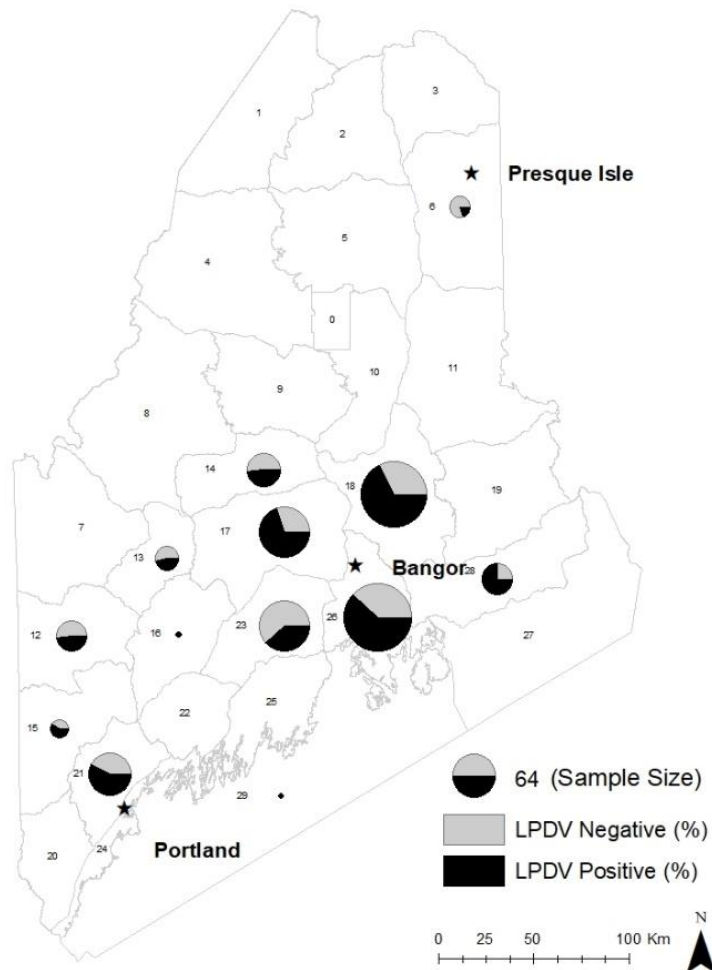


Figure 4.1. Lymphoproliferative disease virus prevalence by wildlife management district in 699 wild turkeys collected 2017–2020 in Maine.

We collected tarsometatarsus bones from hunter-harvested ($n = 72$) wild turkeys during the spring male-only hunting season (April–June). We captured live wild turkeys using rocket nets during three winter seasons (Dec/Jan–March, 2018–2020) and collected whole blood either (1) from the brachial vein into a blood tube containing EDTA (1–5 mL; $n = 263$) or (2) from a foot venipuncture into a capillary tube and stored in Queen’s lysis buffer (~1mL; $n = 363$). In addition, we obtained one bone marrow sample from a live-captured individual post-mortem. We recorded information pertaining to outward disease symptoms when observed. We recorded GPS points at the capture site location for live-captured wild turkeys and at the center of the town of harvest for hunter-harvested wild turkeys to discern WMD and Maine Department of Inland Fisheries and Wildlife Regions (hereafter regions) of collection for all sampled birds. All capture, handling, and sampling of wild turkeys was approved by the University of Maine Institutional Animal Care and Use Committee (IACUC Protocol # A2017_11_03).

Laboratory Methods

We amplified a 413 base pair region of the retroviral *gag* gene (partial p31 and Capsid) for LPDV detection. All sample processing, DNA extraction, and pathogen diagnostics for LPDV detection followed the methods described in Shea et al. (*in review*). Additionally, all positive PCR products were cleaned using Exonuclease 1 and Shrimp Alkaline Phosphatase (ExoSAP-IT; Applied Biosystems, Foster, CA) following manufacturer’s instructions for cycling conditions, and sequenced in both forward and reverse directions on a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) at the University of Maine DNA Sequencing Facility.

Analytical Methods

We performed all downstream LPDV proviral sequence editing and alignment in Geneious 2020.1.1 (<https://www.geneious.com>) for 409 LPDV-positive individuals. We used the

Geneious alignment tool to perform a pairwise alignment of the forward and reverse directions of each individual to obtain consensus sequences. We identified ambiguous bases in 45 individuals (11%) and estimated haplotypes using PHASE 2.1.1 (Stephens et al. 2001), which allowed haplotype prediction for 28 of these sequences with >90% certainty. Therefore, we omitted 17 sequences for which the ambiguous bases could not be resolved from all analyses. We performed a multiple alignment of the consensus sequences using the MUSCLE plugin (Edgar 2004) in Geneious. We reduced the dataset to unique haplotypes for phylogenetic analysis. For further comparison, we accessed 7 additional sequences sourced from GenBank from different United States or Canadian Provinces (Arkansas, Colorado, Allison et al. 2014; West Virginia, Thomas et al. 2015; New York, Alger et al. 2017; Manitoba, Quebec, Ontario, MacDonald et al. 2019b, 2019a). Those from Manitoba, Ontario, New York, and West Virginia were from asymptomatic wild turkeys while those from Quebec, Arkansas, and Colorado were from diagnostic cases. Accession numbers for all sequences sourced from GenBank are listed in Table 4.1.

Table 4.1. Accession numbers, locations, and associated references for all lymphoproliferative disease virus *gag* gene sequences obtained from NCBI GenBank.

Accession number	Location	Reference
KC802224	Arkansas	Allison et al. 2014
KC801953	Colorado	
KP299680	West Virginia	Thomas et al. 2015
KU211641	New York	Alger et al. 2017
MK548372	Manitoba	MacDonald et al. 2019a
MK548383	Quebec	
MF953384	Ontario	MacDonald et al. 2019b
U09568	Israel	Sarid et al. 1994

We used DnaSP (version 6) to estimate the number of unique haplotypes (h), the haplotype (gene) diversity (H_d), and nucleotide diversity (π) diversity of the 419 sequences from wild turkeys in Maine (Librado and Rozas 2009; Rozas et al. 2017). Haplotype diversity is a measurement that reflects both the number and frequency of haplotypes in the population (Nei 1987). Nucleotide diversity assesses variation in polymorphisms within the population, by computing the average number of nucleotide differences per site in all pairwise sequence comparisons. (Nei and Li 1979; Nei 1987). Gaps/missing information were excluded only in pairwise comparisons that contained them. One sequence contained an indel, one sequence had data missing for the first two base pairs, and one sequence had data missing for the last 32 base pairs. Additionally, we used Geneious to estimate percent pairwise identity (the opposite of nucleotide diversity) and percent nucleotide identity (percent identical sites). We also assessed percent nucleotide identity, between pairwise comparisons of a random recently divergent Maine haplotype (haplotype 173) with both the Israeli prototype (U09568, domestic turkey from Israel; Sarid et al. 1994) and North American prototype (KC802224, wild turkey from Arkansas; Allison et al. 2014) in Geneious.

MRBAYES version 3.2.7 (Huelsenbeck and Ronquist 2001) was employed to reconstruct evolutionary relationships among 229 unique LPDV haplotypes found in wild turkeys in Maine and 7 unique haplotypes from outside of Maine. MRBAYES relies on Bayesian inference and uses Metropolis-coupled Monte Carlo (MCMC) methods (Geyer 1991) to estimate the posterior distribution of model parameters. The nucleotide substitution model that best fit our data was the GTR+G+I designation indicated by the modelTest function (phangorn package, Schliep 2011) in Program R (R Core Team 2021), comparing models using AIC model selection (Figure B.1). However, this model is the most complex model and experienced issues converging and we did

not intend to include the parameter for proportion of invariant sites because the parameter for variation rate across sites (gamma distribution) should account for slowly evolving sites (Yang 2014). Therefore, we instead chose the less parameter-rich HKY + G model, which had similar model support, to ensure convergence (Figure B.1). We designated a LPDV sequence from Manitoba, Canada as the outgroup, which was found to be divergent based on preliminary analysis. In MRBAYES, we ran two simultaneous independent runs, each containing 1 cold chain and 3 hot chains for MCMC sampling of the parameter space, and applied an unconstrained molecular clock. We ran the model for 12 million generations and sampled the parameter posterior distributions every 100 generations, assessing model convergence by examining the average standard deviation of split frequencies (< 0.01), the Potential Scale Reduction Factor (≈ 1.0 ; Table B.1), as well as by visualizing posterior traces in Tracer v.1.7.1 (Rambaut et al. 2018) to ensure effective sample sizes (ESS) for all parameter estimates exceeded 200. Parameter estimates and a consensus tree were estimated using the combined posterior distributions from the two independent runs, after discarding the first 25% as burn-in. We also replicated this run to verify parameter estimation.

We used Program R (R Core Team 2021) for further analysis of our final phylogenetic tree to associate individual sequences with geographic locations (WMD). We also estimated the prevalence and spatial distribution of each haplotype in our sample.

We employed GenAlEx version 6.5 (Peakall and Smouse 2006, 2012) to conduct a Principal Coordinates Analysis (PCoA; Torgerson 1958) to examine genetic structure. We analyzed the pairwise genetic distances between each sequence ($n = 420$), designating the data as haploid and using squared genetic distances in the genetic distance calculation. We applied the

covariance-standardized PcoA method on the genetic distance matrix, and color-coded each sequence based on geographic location (WMD or Region).

We conducted a Mantel's test (Mantel 1967) in GenAlEx v. 6.5 to determine whether there was genetic isolation by distance. The null hypothesis of the Mantel's test is that genetic distance matrix is not correlated with the geographic distance matrix. For this analysis, we only used sequences from live captured turkeys with known capture locations, and omitted sequences from hunter-harvested individuals (59), for which we were only able to assign locations to the town level. Therefore, the Mantel's test assessed matrix correlation between geographic (capture site location in decimal lat/long) and linear genetic distances of 361 sequences using 999 permutations.

Results

We detected LPDV in 409 out of 699 (59%) wild turkeys (Figure 4.1) and used these viral sequences for downstream analysis. All individuals were outwardly asymptomatic, except for one that sustained a head lesion. After omitting 17 individuals whose ambiguous bases could not be resolved with enough certainty, 392 individuals remained, 364 of which contained a single haplotype and 28 of which contained two haplotypes for a total of 420 sequences. We identified 229 unique haplotypes from wild turkeys in Maine with a high haplotype diversity of 0.982. The majority (73%) of haplotypes were found in single wild turkeys (Figure 4.2). Nucleotide diversity was 0.015 (1.5%), with 98.5% pairwise identity, in 420 sequences, with an average of 6.3 nucleotide differences across 412.9 sites in pairwise comparisons. The percent identical sites was 58.4%. The percent identical sites between the North American prototype (Arkansas diagnostic case, 2009; Allison et al. 2014) and Maine haplotype 173 was 93.5% while the percent identical sites between the Israeli prototype and Maine haplotype 173 was 86.7%. In 409

LPDV-positive wild turkeys, we detected 45 individuals (11%) that contained LPDV sequences with ambiguous bases, which may reflect either the introduction of mutations during host genome integration or infection of a host by multiple viral strains.

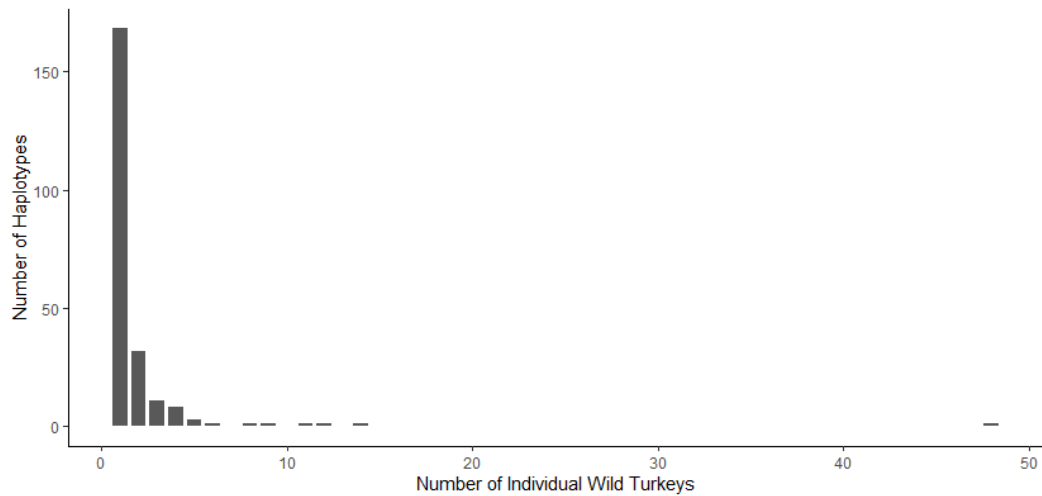


Figure 4.2. Distribution of the number of lymphoproliferative disease virus haplotypes by the number of wild turkeys in Maine (2017–2020) that a given LPDV haplotype infects.

The summarized parameter values of the phylogeny after discarding a 25% burn-in are shown in Table B.1 (Ripley 1987; Gelman and Rubin 1992), confirmed by the replicated run (data not shown). The LPDV consensus phylogeny revealed high lineage diversity. The posterior node probabilities, particularly at ancestral nodes, was generally low (Figure 4.3), indicating that the topology of the tree and evolutionary relationships could often not be resolved given our data. We colored terminal branches by WMD for 197 haplotypes that were only found in one WMD (86%; colored branches, Figure 4.3). The remaining 32 haplotypes (14%; black branches, Figure 4.3) were found in wild turkeys distributed across multiple WMDs. The geographic distribution of haplotypes that were found in at least 10 wild turkeys distributed across more than one WMD are presented (Figure 4.3).

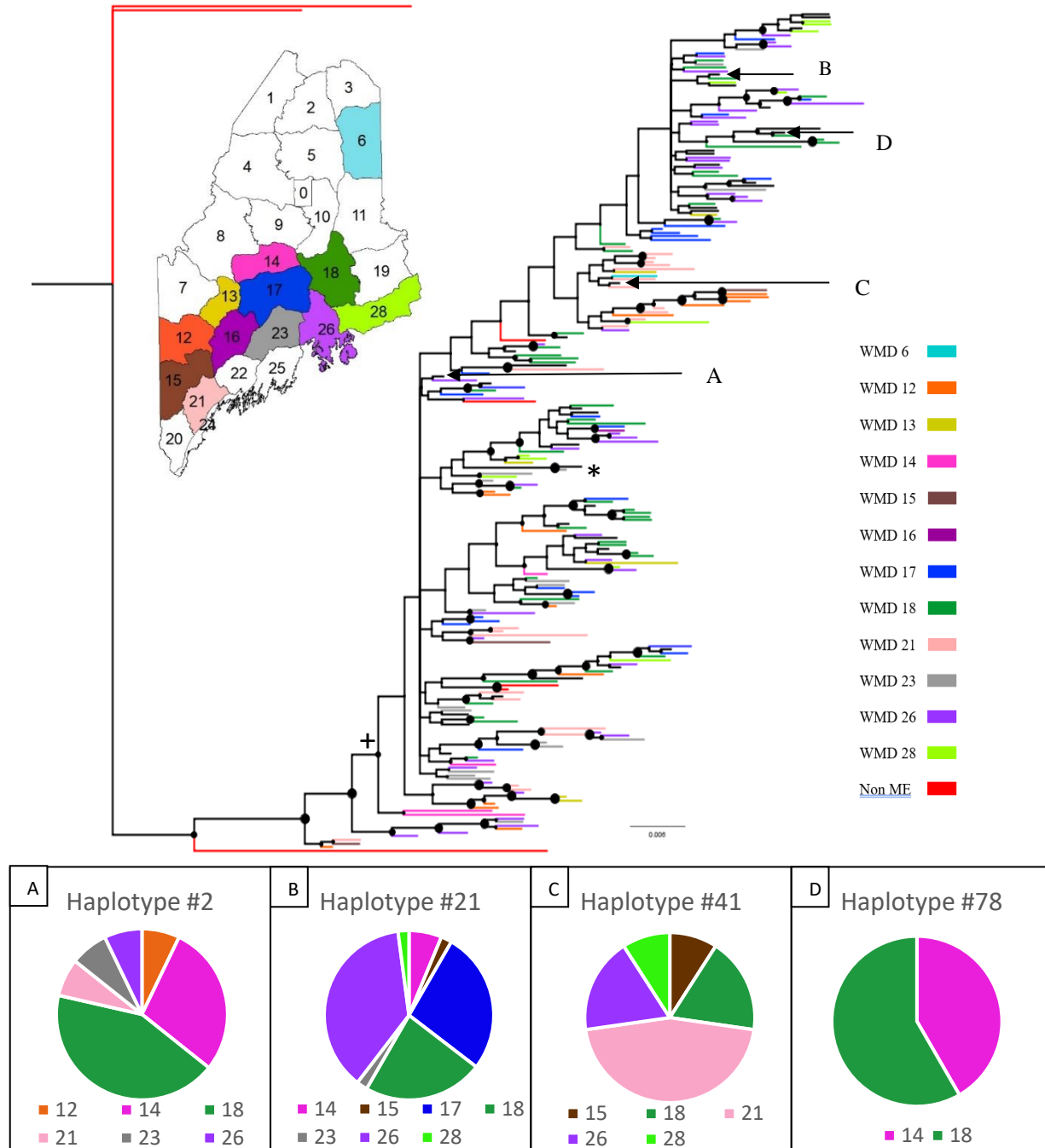


Figure 4.3. Lymphoproliferative disease virus (LPDV) consensus phylogeny using viral haplotypes derived from wild turkeys sampled between 2017–2020 in Maine. The evolutionary relationships among unique LPDV sequences ($n = 229$) found in 392 wild turkeys in Maine were reconstructed with respect to 7 sequences from wild turkeys from other states/provinces in North America. Branch lengths are proportional to the number of nucleotide substitutions. The

outgroup was designated as the isolate from Manitoba. Posterior node probability (PNP) is represented by the size of the node circles, with a star denoting an example of a node with PNP of 1 and a plus sign denoting an example of a node with PNP of 0.5. Terminal branch color corresponds to wildlife management district (WMD). Branches of haplotypes found in turkeys distributed across more than one WMD remain black. Branches of sequences from outside of Maine are colored red. Haplotypes found in at least 10 wild turkeys are shown via pie charts, with colors representing their spatial distribution across WMDs: (A) 14 sequences of haplotype #2, (B) 48 sequences of haplotype #21, (C) 11 sequences of haplotype #41, and (D) 12 sequences of haplotype #78.

The PCoA revealed some clustering, with the first two principal component axes explaining 22.76% of the variation in pairwise squared genetic distance, suggestive of genetic structure. However, this genetic structure is not explained by geographic location when visualized by WMD or Region (Figures 4.4 and 4.5, respectively).

The Mantel's Test demonstrated a significant positive relationship between the geographic distance and linear genetic distance matrices ($p = 0.033$; Figure 4.6). This provides some evidence for divergence among LPDV strains at finer spatial scales, however the effect size was weak ($\beta = 0.0007$) and geographic distance explained a small portion of the variation in linear genetic distance ($R^2 = 0.004$).

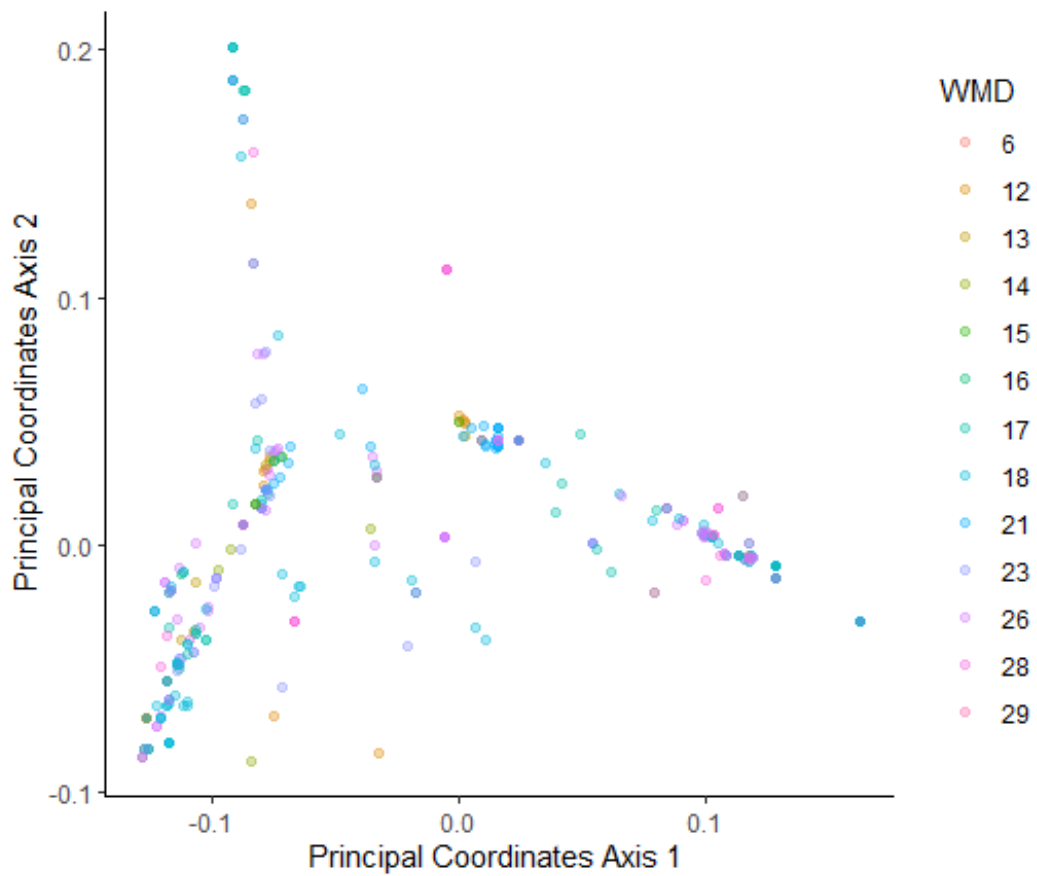


Figure 4.4. Principal coordinate analysis of the pairwise genetic distance matrix of lymphoproliferative disease virus, colored by wildlife management district, infecting wild turkeys collected between 2017–2020 in Maine. The first two principal axes are shown, which explain 22.76% of the observed genetic variation.

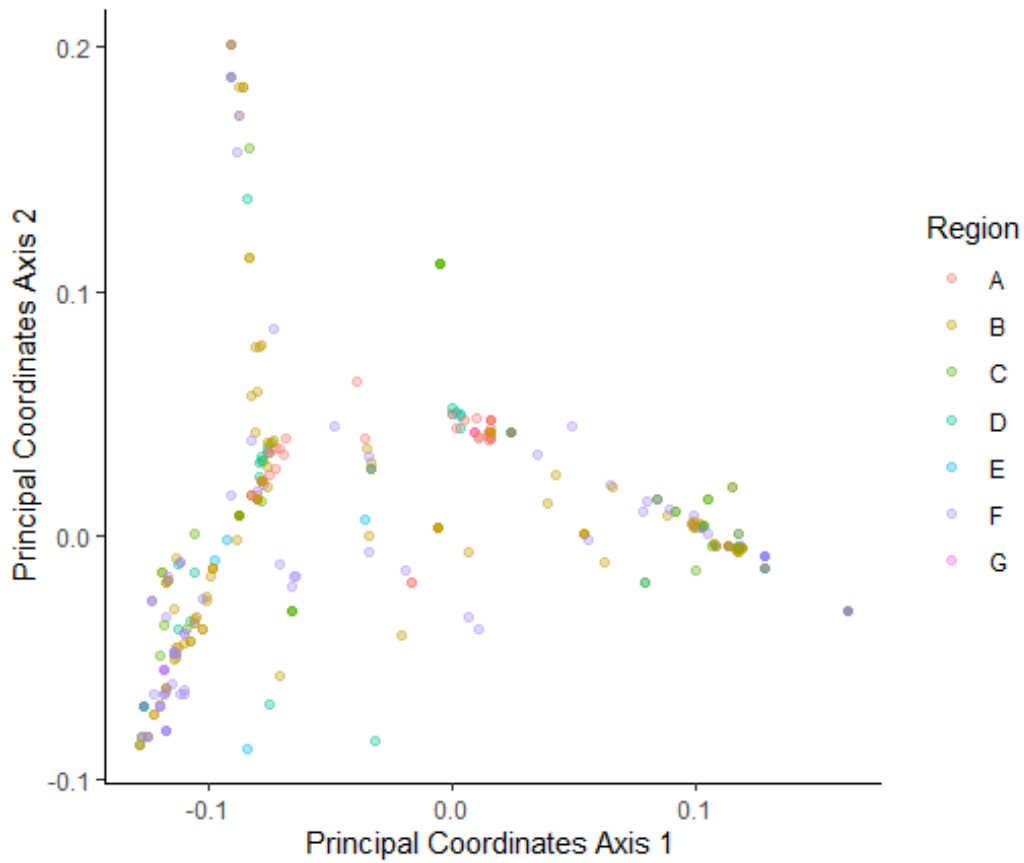


Figure 4.5. Principal coordinate analysis of the pairwise genetic distance matrix of lymphoproliferative disease virus, colored by region, infecting wild turkeys collected between 2017–2020 in Maine. The first two principal axes are shown, which explain 22.76% of the observed genetic variation.

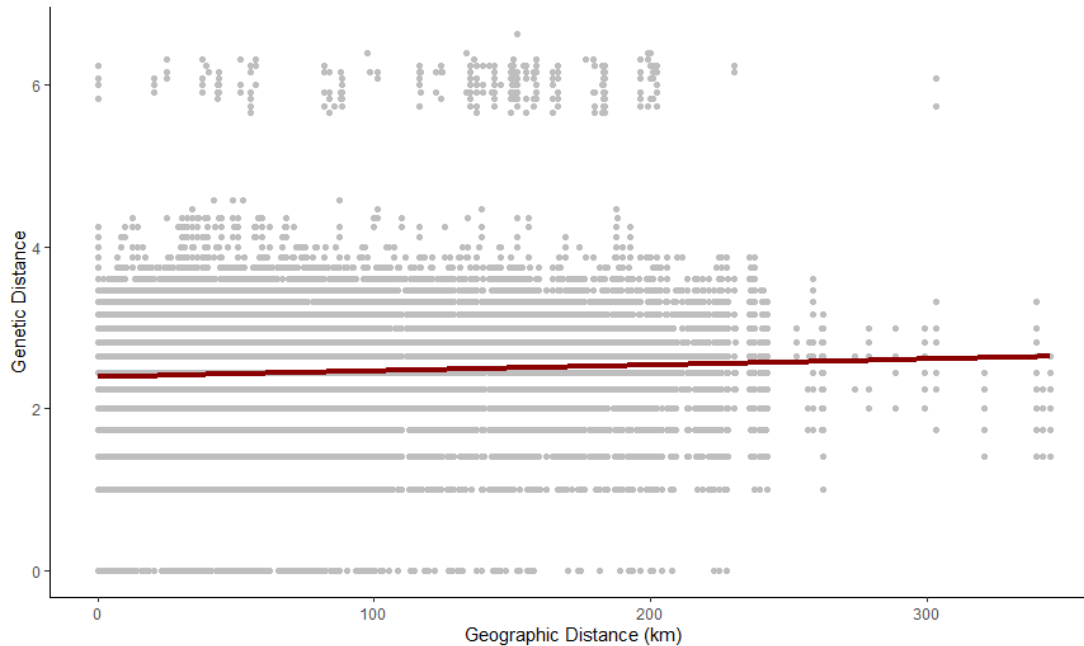


Figure 4.6. Mantel’s test assessing the relationship between geographic distance and linear genetic distance in pairwise comparison of 361 sequences from 339 live-captured wild turkeys sampled from 2018–2020 in Maine.

Discussion

Our study is the first to examine genetic variation of LPDV at finer spatial scales to better understand transmission of the virus in wild turkeys. We observed a high genetic diversity of LPDV strains, with over half of the strains being unique sequence haplotypes that were not found in more than one bird. Our phylogenetic analyses also revealed low node support for clustering of genetic lineages, which follows previous findings that RNA viruses commonly maintain extremely high genetic heterogeneity (Duffy et al. 2008; Duffy 2018). Furthermore, we found a lack of spatial structure in LPDV strains infecting wild turkeys across the state. However, we did

find evidence for a weak genetic isolation by distance signal, with a positive correlation between geographic and LPDV genetic distance, although this explained very little of the variation in genetic distance. Taken together, the high diversity of LPDV strains and lack of spatial structuring suggests that there is high geographic mixing and connectivity of wild turkey hosts and their pathogens (Lauring 2020).

Viral genetic heterogeneity can reveal information on transmission and infection stage, much of which has yet to be studied in LPDV in natural ecosystems. Other RNA retroviruses, such as human immunodeficiency virus (HIV) initially reside in hosts as a group of related viral variants at the infective stage and transmission to another host can result in a bottleneck effect (transmission bottleneck), reducing the variation in the initial viral pool (McNearney et al. 1992; Ahmad et al. 1995). Furthermore, sequence diversity has been found to be higher in samples collected at later stages of HIV infection compared with the initial stages (McNearney et al. 1992). This may suggest that particular variants more readily persist and establish in new hosts during transmission (McNearney et al. 1992; Leitner and Romero-severson 2018) with potential successive explosive viral replication following initial infection (as seen in HIV; Lauring 2020), which could explain the high genetic heterogeneity apparent in our study. If a substantial amount of within host evolution occurs prior to transmission, we may be missing a lot of the variation necessary for reconstructing transmission networks. High genetic diversity could offer the virus an opportunity to evolve towards higher infectivity and transmissibility, increasing the risk of outbreaks and altering the observable effects of infection on the host.

Mutations may arise at any stage of the retroviral life cycle including during viral replication, transcription, integration, or proviral replication by the host (Roberts et al. 1988; Clavel et al. 1989), which promotes high genetic heterogeneity in retroviruses. Reverse

transcriptase is an enzyme used by retroviruses to convert RNA to complementary single-stranded DNA, and then into double-stranded DNA for integration into the host genome. These stages are prone to high retroviral mutagenesis because reverse transcriptase is highly error-prone (Roberts et al. 1988; Garcia-Diaz and Bebenek 2007; Duffy et al. 2008). Proviral replication by the host utilizes DNA polymerase, which is much less error-prone, but variation still exists in mutational capacity (Kunkel 2004; Renner and Szpara 2018). Additionally, the per-genome mutation rate has been found to remain relatively constant across viruses, resulting in faster mutation rates for small viruses compared with large viruses (Sanjuán and Domingo-Calap 2016); LPDV has a relatively small genome comprised of only 7,432 nucleotides (Allison et al. 2014).

During viral replication within hosts, genetic variation can be introduced by mutation and recombination. In another retrovirus, comparison of replication-competent viral variants to the integrated proviral DNA in a tissue culture system revealed that the genetic makeup of the proviral DNA was a result of recombination of the original variants during replication (Clavel et al. 1989). Genetic recombination can occur between variants of the same retrovirus, or between/among variants of different retroviruses (gene transfer) within a host, as demonstrated with avian leukosis virus (ALV) and sarcoma viruses, which are closely related to LPDV (Blair 1977; Clavel et al. 1989; Allison et al. 2014). Lymphoproliferative disease virus is replication-competent, but lacks an oncogene (Payne and Venugopal 2000; Allison et al. 2014), meaning it is self-sufficient for viral replication, but requires host cell machinery to induce oncogenesis. Since it is replication competent, the opportunity likely exists for recombination of LPDV variants, as well as between LPDV and other retroviruses, such as reticuloendotheliosis virus (REV), which have simultaneously been found in the same host (Shea et al. *in review*; Blair

1977; MacDonald et al. 2019b). These processes common to retroviruses may explain the high diversity of LPDV in our study, but further investigation is warranted to formally evaluate the role of recombination in the evolution of LPDV.

Aside from our observation of overarching viral genetic diversity in wild turkeys in Maine, we also identified 11% of individuals with ambiguous nucleotide sequences. This might indicate that either a mutation occurred during the conversion of RNA to DNA, or during replication. Alternatively, it is possible that two strains inserted into different locations within the host genome, which is supported by previous findings that demonstrated three cloned LPDV proviruses originated from different sites within the domestic turkey genome (Chajut et al. 1991; Biggs 1997). Allison et al. (2014) identified the insertion site of the North American prototype strain and found that it integrated near a known host oncogene, which is required for cell transformation by LPDV. It remains unknown whether this insertion site is common for LPDV in wild turkeys, or, similar to domestic turkeys, if LPDV inserts randomly. We were able to resolve many of these ambiguities using a phasing algorithm and considered these as distinct variants in the viral population, but future research is needed to better understand how LPDV interacts with the host genome to influence infection and transmission dynamics.

Pathogen transmission dynamics across host spatial and social networks could be strain-specific (Fountain-Jones et al. 2017). We sought to identify spatial structure of LPDV strain diversity within wild turkeys in Maine, however, high genetic diversity and a phylogeny with low ancestral node support made it difficult to reconstruct historical relationships among lineages. The phylogeny annotated by geography showed a lack of support for spatial structure, and the PCoAs indicated that although there was some structure to the genetic data, it was not explained by WMD or Region of sampling. However, the Mantel's test identified a positive,

albeit weak relationship between geographic and genetic distances at a finer spatial scale, suggesting viral genetic divergence may be occurring, but only within the very recent past.

The apparent high rate of evolution and lack of spatial structure could be a result of potential aforementioned intrahost evolution, but also could be a reflection of wild turkey social and spatial structure. Fountain-Jones et al. (2017) identified distance between lion prides as a significant factor in explaining FIV retroviral strain network structure. This may suggest that when the distance between groups is diminished, there is less pathogen strain structure in the system. Wild turkeys are gregarious birds that form large dynamic winter flocks (Watts and Stokes 1971; Healy 1992), and then disperse widely in spring as they mate and search for adequate nesting habitat (Niedzielski et al. 2016). Their spring home range size has been found to be higher at their northern range limit (Niedzielski et al. 2016), which may indicate higher contact rates than in other geographic regions. With large annual home range sizes and gregarious dynamic flock groupings, high wild turkey connectivity across the landscape could result in increased viral exposure and spread across the state (59% prevalence), resulting in general ubiquitous transmission and subsequent lack of viral genetic spatial structure. The somewhat low explanation of variation in genetic distance exhibited by the two principal components of the PCoA, likely signifies multiple factors are contributing to shaping the genetic diversity of LPDV in wild turkeys.

Viral genetic diversity and spatial patterns, or lack thereof, are often associated with dynamics regarding establishment and spread of pathogens within the host pathogen system. High diversity of dengue virus in Singapore is reportedly a reflection of the multiple viral introduction events of different strains into Singapore followed by evolution within Singapore (Lee et al. 2012). Lineages of dengue virus initially maintained localized spatial structure at the

point of introduction, but subsequent host interactions and spread ultimately resulted in no clear spatial differentiation or clustering among strains (Lee et al. 2012). Similarly, rapid wild turkey population growth, which has been documented following reintroduction (Allen 2000; Sullivan 2017), is a likely explanation for the lack of spatial structure in LPDV genetic diversity. For example, as cougar populations recovered in size in western North America, the FIV retroviral pathogen also experienced growth in population size and spatial expansion, which reduced the spatial structure of the virus (Biek et al. 2006). The reintroduction of 111 wild turkeys from Vermont and Connecticut into Maine in 1977–1978 and 1987–1988, respectively (Allen 2000) could have resulted in either the initial introduction of LPDV or the reintroduction of divergent LPDV strains from different sources into Maine’s wild turkey population. It is generally assumed that LPDV is endemic in wild turkey populations and has been previously undetected in the past, though this is not empirically confirmed (Allison et al. 2014; Thomas et al. 2015). The relocation of wild turkeys from multiple source locations during reintroduction events in Maine, which may have already been a product of previous mixing due to national reintroduction and translocation campaigns, may be a likely explanation for the high diversity observed by unnaturally spreading diverse lineages across the continent. Comparing LPDV sequence data among wild turkeys in Connecticut, Vermont, and Maine may reveal insights into the phylogenetic relationship among the source and translocated populations. However, it is possible that rapid viral evolution and host mixing since reintroductions may overshadow the deep phylogenetic relationship without substantial temporal sampling and large sample sizes.

If wild turkey movement ecology contributes largely to the transmission, evolution and diversity of LPDV, then as a non-migratory species with large home range sizes, genetic structure of LPDV may only be apparent at moderate to large spatial scales. While this was not

an objective of our study, we did incorporate seven sequences from different states/provinces outside of Maine to assess divergence between a high density sample of LPDV sequences within the state and sequences distributed over a broader spatial scale. Despite this, we found no discernable spatial pattern, though the sequences from Arizona and Manitoba were divergent from all others. Allison et al. (2014) and Thomas et al. (2015) performed phylogenetic analysis of LPDV sequences collected between 2009-2012 from wild turkeys distributed across 18 eastern states and their results similarly revealed low node support and high diversity, but with some spatial clustering by state; though, resolution of this analysis may have been hindered by small sample sizes by state. Higher resolution viral genome data and temporal sampling may be required to resolve the transmission and evolutionary dynamics of LPDV, given its high diversity and recent large increase in population size due to reintroduction events across the continent. Furthermore, while we may have sampled a wild turkey from a particular WMD, as free-ranging wild animals, they are free to move among and between WMD. So, it is possible that our specific delineations do not capture typical turkey movement and connectivity. Therefore, it would be useful to additionally couple high resolution viral genome data with turkey movement data to get a better picture of transmission pathways across spatial scales.

It is possible that grouping genetic data by ecological arbitrary regions (i.e., states or WMDs), reduces the ability to detect spatial structure in the genetic data. Using arbitrary delineations, such as states or WMDs, may result in two viral strains being geographically close together (i.e., on the border of two WMDs), but still placed into discrete geographic boundaries, reducing the geographic resolution and obscuring any potential spatial structure. Our detection of a geographic signal when assessing the variation in pairwise genetic distances supports this hypothesis. While it was not a particularly strong correlation, it does suggest weak spatial

structure of LPDV in Maine that we were not able to capture when grouping by WMD. In addition, incorporating landscape components may elucidate factors impacting host connectivity and transmission dynamics. For instance, two distinct lineages of FIV in Montana cougars were spatially structured in association with an interstate highway (Wheeler et al. 2010), which could have been overlooked if investigated at a larger spatial scale.

Comparison of the genomes of the North American prototype (wild) compared to the Israeli prototype (domestic) revealed 87.9% identical sites (Allison et al. 2014). A comparison of the *gag* region of one of our more recently diverged Maine haplotypes demonstrated a similar percent identity to the Israeli prototype (86.7%), which was lower than when compared to the North American prototype (93.5%). This seems appropriate since only a few sequences from South Carolina were found to group with the Israeli prototype, suggesting a lack of spread of this particular lineage (Allison et al. 2014). The percent identity of the North American prototype (wild) compared to the Israeli prototype (domestic) of the *gag* (88.3%), *pro* (88.3%), and *pol* (90.1%) regions were slightly higher, while *env* was slightly lower (86.6%) across the entire genomes (Sarid et al. 1994; Allison et al. 2014).

We specifically assessed the evolution and diversity of a 413 base pair region spanning the partial p31 and partial capsid genes of the *gag* region. The *gag* region of retroviruses typically encodes proteins that are typically responsible for the development of immature viral-like particles (Freed 2004; Ako-Adjei et al. 2005). Specifically, the proteins p31 and capsid are considered to be two major virion (infective form of virus) structural proteins in LPDV in domestic turkeys (Gazit et al. 1986). Genetic variation in the *gag* region of HIV was found to correlate with differences in cytotoxic T cell recognition of the host in particular cell types, suggesting evolution of host evasion (Phillips et al. 1991). Thus, we targeted a region of the

genome that may experience average, or slightly higher than average substitution rate and potential selective pressure, compared to other genes within the genome. However, it is also important to consider that there are differences among retroviruses, and even alpharetroviruses, in the location (region), function, and expression of retroviral genes (Justice IV and Beemon 2013; Allison et al. 2014). For instance, the protease gene is not expressed in the *gag* region in LPDV as it is in other alpharetroviruses, such as ALV (Justice IV and Beemon 2013; Allison et al. 2014). Thus, research confirming characteristics of these genes in LPDV will help understand the potential variation in selective pressures acting across the viral genome. Lymphoproliferative disease virus may be under substantial evolutionary pressure driven by molecular interactions between the viral genome and specific host cells. For instance, ‘selective sweeps’ occur in influenza A virus when directional selection results in new variants continuously arising and replacing previously existing variants (Kosakovsky Pond et al. 2008; Klingen et al. 2018). Whether or not the LPDV *gag* gene or other regions of the genome are associated with evasion of the host immune response in wild turkeys or is undergoing selective pressure has not been addressed to our knowledge and should be considered a priority in future research.

In conclusion, we present four years of temporal data that reveal high LPDV strain diversity at the smaller, state-wide scale in Maine’s wild turkey, data which mirrors results found at large spatial scales (Allison et al. 2014; Thomas et al. 2015). The high diversity, low posterior node probabilities, and lack of genetic spatial structure may reflect intrahost evolution, widespread mixing of viral strains across the state, or the history of wild turkey translocation and demographic expansion. Future host and pathogen whole genome sequence data is required to increase genetic resolution for further investigation into LPDV transmission dynamics in wild

turkeys. These data will be critical for identifying hotspots and pathways of transmission and assessing spillover risk to other wild and domestic birds.

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APPENDIX A: Chapter 3 Supplemental Material

Table A.1. Initial AIC model selection comparing univariate models to identify relevant non-pathogen variables affecting weekly survival rate of female wild turkeys captured and monitored from 2018–2020 in Maine. Season was kept as a baseline null model in the second AIC model selection step.

Model	<i>k</i>	AICc	ΔAICc	Weight	Dev.
Season	4	930.940	0.000	0.778	922.933
Null	1	934.614	3.674	0.124	932.613
Age	2	936.396	5.455	0.051	932.393
Trans.Type	2	936.555	5.615	0.047	932.553

Table A.2. Second AIC model selection to identify pathogen variables affecting weekly survival rate of 163 female wild turkeys captured and monitored from 2018–2020 in Maine. All models except the intercept-only model contain season as an explanatory variable, following Table S1. Variables determined to be significant upon interpretation of coefficients and their 95% confidence intervals in a model containing all supported variables ($<2 \Delta$ AICc) are italicized.

Model	<i>k</i>	AICc	ΔAICc	Weight	Dev.
Season + <i>REV</i>	5	928.618	0.000	0.324	918.607
Season + Age + <i>REV</i>	6	930.215	1.598	0.146	918.200
Season	4	930.940	2.322	0.101	922.933
Season + <i>Coinf</i>	7	931.260	2.642	0.086	917.239
Season + <i>LPDV</i>	5	931.429	2.811	0.079	921.418
Season + Age + <i>REV</i> + <i>REV</i> *Age	7	931.797	3.179	0.066	917.776
Season + Age	5	932.413	3.795	0.049	922.402
Season + Age + <i>Coinf</i>	8	933.149	4.531	0.034	917.123
Season + Age + <i>LPDV</i>	6	933.270	4.652	0.032	921.254
Season + <i>REV</i> + <i>REV</i> *Season	8	933.563	4.945	0.027	917.537
Season + Age + <i>LPDV</i> + <i>LPDV</i> *Adult	7	934.087	5.469	0.021	920.067
Null	1	934.614	5.996	0.016	932.613
Season + <i>LPDV</i> + <i>LPDV</i> *Season	8	935.393	6.775	0.011	919.366
Season + Age + <i>Coinf</i> + <i>Coinf</i> *Age	11	936.434	7.816	0.006	914.386
Season + <i>Coinf</i> + Season * <i>Coinf</i>	16	938.959	10.341	0.002	906.858

Table A.3. Initial AIC model selection comparing univariate models to identify relevant non-pathogen variables affecting daily nest survival rate of female wild turkeys captured and monitored from 2018–2020 in Maine. Nest age was kept as a baseline null model in the second AIC model selection step.

Model	<i>k</i>	AICc	ΔAICc	Weight	Dev.
Nest.Age	2	535.244	0.000	1.000	531.238
Null	1	553.170	17.926	0.000	551.168
Turkey.Age	2	554.053	18.809	0.000	550.048
Nest.Attempt	3	554.113	18.870	0.000	548.103
Nest.Initiation	2	555.035	19.791	0.000	551.030
Trans.Type	3	556.775	21.531	0.000	550.764
Nest.Year	3	557.157	21.913	0.000	551.147

Table A.4. Second AIC model selection models to identify pathogen variables affecting daily nest survival of female wild turkeys captured and monitored 2018–2020 in Maine. All models except the intercept-only model contain nest age as an explanatory variable, following Table S3. Variables determined to be significant upon interpretation of coefficients and their 95% confidence intervals in a model containing all supported variables ($<2 \Delta$ AICc) are italicized.

Model	<i>k</i>	AICc	ΔAICc	Weight	Dev.
<i>Nest.Age + T.Age</i>	3	534.828	0.000	0.204	528.817
Nest.Age	2	535.244	0.416	0.166	531.238
<i>Nest.Age + T.Age + LPDV + LPDV * T.Age</i>	5	535.271	0.443	0.164	525.244
<i>Nest.Age + T.Age + REV</i>	4	536.480	1.652	0.089	528.462
<i>Nest.Age + T.Age + LPDV</i>	4	536.777	1.949	0.077	528.759
<i>Nest.Age + REV</i>	3	536.830	2.002	0.075	530.820
<i>Nest.Age + LPDV</i>	3	537.124	2.296	0.065	531.113
<i>Nest.Age + T.Age + Coinf</i>	6	537.653	2.826	0.050	525.616
<i>Nest.Age + T.Age + REV + REV*T.Age</i>	5	537.877	3.050	0.044	527.851
<i>Nest.Age + Coinfection</i>	5	537.923	3.095	0.043	527.896
<i>Nest.Age + T.Age + Coinf + Coinf* T.Age</i>	9	539.241	4.413	0.022	521.162
Null	1	553.170	18.342	0.000	551.168

Table A.5. Clutch size overall or by age or lymphoproliferative disease status for first, second, and third nest attempts with standard errors for 107 wild turkey nests over three nesting seasons (2018–2020) in Maine.

Cohort	Nest Attempt	Clutch Size	SE	Sample Size
All	All Combined	11.5	0.2	107
	First	11.8	0.2	92
	Second	9.9	0.5	14
	Third	8	NA	1
Adult	All Combined	11.6	0.2	99
	First	11.8	0.2	86
	Second	10.2	0.5	12
	Third	8.0	NA	1
Juvenile	All Combined	10.0	1.1	8
	First	10.5	1.3	6
	Second	8.5	1.5	2

Table A.6. Initial AIC model selection comparing models to identify non-pathogen variables affecting clutch size during the first nesting attempt of female wild turkeys captured and monitored from 2018–2020 in Maine. Nest initiation and nest initiation quadratic term were kept as a baseline model in the second AIC model selection step.

Models	<i>k</i>	AICc	ΔAICc	ModelLik	Weight	LL
Nest.Init	2	369.487	0.000	1.000	0.730	-181.607
Nest.Init ²	3	371.481	1.994	0.369	0.270	-181.510
Weight	2	416.652	47.165	0.000	0.000	-205.190
Null	1	416.961	47.475	0.000	0.000	-206.413
Age	2	417.117	47.630	0.000	0.000	-205.422
Nest.Year	3	421.091	51.604	0.000	0.000	-206.315

Table A.7. Second AIC model selection models to identify pathogen variables affecting clutch size during the first nesting attempt of female wild turkeys captured and monitored from 2018–2020 in Maine. All models contain nest initiation and nest initiation quadratic term as explanatory variables, following Table S6 (for simplification, we only show the quadratic term). Variables determined to be significant upon interpretation of coefficients and their 95% confidence intervals in a model containing all supported variables ($<2 \Delta AICc$) are italicized

Model	<i>k</i>	AICc	$\Delta AICc$	ModelLik	Weight	LL
Nest.Init ² + <i>LPDV</i>	4	362.656	0.000	1.000	0.411	-175.979
Nest.Init ² + <i>LPDV</i> + Age	5	363.169	0.512	0.774	0.318	-175.090
Nest.Init ² + <i>LPDV</i> + Age + <i>LPDV</i> *age	6	365.404	2.748	0.253	0.104	-175.036
Nest.Init ² + <i>Coinf</i>	6	365.976	3.320	0.190	0.078	-175.321
Nest.Init ² + <i>Coinf</i> + Age	7	366.248	3.591	0.166	0.068	-174.256
Nest.Init ² + <i>Coinf</i> + <i>Coinf</i> *Age	9	369.970	7.313	0.026	0.011	-173.627
Nest.Init ²	3	371.481	8.824	0.012	0.005	-181.510
Nest.Init ² + Age	4	373.169	10.513	0.005	0.002	-181.236
Nest.Init ² + <i>REV</i>	4	373.176	10.519	0.005	0.002	-181.239
Nest.Init ² + <i>REV</i> + Age	5	374.915	12.258	0.002	0.001	-180.963
Nest.Init ² + <i>REV</i> + Age + <i>REV</i> *Age	6	376.991	14.334	0.001	0.000	-180.829
Null	1	416.961	54.305	0.000	0.000	-206.413

Table A.8. Initial AIC model selection comparing univariate models to identify relevant non-pathogen variables affecting clutch size during the second nesting attempt of female wild turkeys captured and monitored from 2018–2020 in Maine. Age was kept as a baseline null model in the second AIC model selection step.

Model	<i>k</i>	AICc	$\Delta AICc$	ModelLik	Weight	LL
Null	1	61.755	0.000	1.000	0.489	-28.332
Age	2	63.566	1.811	0.404	0.198	-27.583
Weight	2	64.295	2.539	0.281	0.137	-27.947
Nest.Init	2	64.386	2.631	0.268	0.131	-27.993
Nest.Init ²	3	67.550	5.795	0.055	0.027	-27.553
Nest.Year	3	68.312	6.557	0.038	0.018	-27.934

Table A.9. Second AIC model selection models to identify pathogen variables affecting clutch size during the second nesting attempt of female wild turkeys captured and monitored from 2018–2020 in Maine. All models contain turkey age as an explanatory variable, following Table S8. No variables were determined to be significant upon interpretation of coefficients and their 95% confidence intervals in a model containing all supported variables ($<2 \Delta AICc$).

Model	<i>k</i>	AICc	$\Delta AICc$	ModelLik	Weight	LL
Null	1	61.755	0.000	1.000	0.533	-28.332
Age	2	63.566	1.811	0.404	0.215	-27.583
Age + LPDV	3	65.053	3.298	0.192	0.102	-26.304
Age + LPDV + LPDV*Age	3	65.053	3.298	0.192	0.102	-26.304
Age + REV	3	66.944	5.189	0.075	0.040	-27.250
Age + REV + REV*Age	4	71.075	9.320	0.009	0.005	-26.788
Age + Coinf	5	74.615	12.860	0.002	0.001	-25.308
Age + Coinf + Coinf*Age	5	74.615	12.860	0.002	0.001	-25.308

Table A.10. Average nest initiation dates for 117 nests by year, overall, and by age for first, second, and third nest attempts of wild turkeys in Maine over 3 nesting seasons (2018–2020).

Cohort	Nest Attempt	Nest Initiation	SE	Sample Size
2018	First	121.8	2.9	20
2019	First	129.3	2.0	32
2020	First	120.5	2.2	47
All	All Combined	128.0	1.6	117
	First	123.6	1.4	99
	Second	151.1	2.6	17
	Third	174.0	NA	1
Adult	All Combined	127.8	1.7	108
	First	123.2	1.5	92
	Second	153.1	2.3	15
	Third	174.0	NA	1
Juvenile	All Combined	130.4	4.1	9
	First	128.9	4.8	7
	Second	136.0	9	2

Table A.11. Initial AIC model selection comparing univariate models to identify relevant non-pathogen variables affecting nest initiation during the first nesting attempt of female wild turkeys captured and monitored from 2018–2020 in Maine. Nest year was kept as a baseline null model in the second AIC model selection step.

Model	k	AICc	ΔAICc	ModelLik	Weight	LL
Nest.Year	3	802.023	0.000	1.000	0.803	-396.799
Null	1	806.160	4.137	0.126	0.101	-401.017
Age	2	807.194	5.171	0.075	0.061	-400.470
Weight	2	808.287	6.265	0.044	0.035	-401.017

Table A.12. Second AIC model selection models to identify pathogen variables affecting nest initiation during the first nesting attempt of female wild turkeys captured and monitored from 2018–2020 in Maine. All models contain nest year as explanatory variable, following Table S11. Variables determined to be significant upon interpretation of coefficients and their 95% confidence intervals in a model containing all supported variables (<2 ΔAICc) are italicized.

Model	k	ΔAICc	Delta_AICc	ModelLik	Weight	LL
<i>Nest.Year</i>	3	802.023	0.000	1.000	0.292	-396.799
Nest.Year + Age	4	803.000	0.978	0.613	0.179	-396.178
Nest.Year + REV	4	803.717	1.695	0.429	0.125	-396.536
Nest.Year + LPDV	4	803.761	1.738	0.419	0.122	-396.558
Nest.Year + LPDV + Age	5	804.436	2.413	0.299	0.087	-395.761
Nest.Year + REV + Age	5	804.743	2.721	0.257	0.075	-395.915
Null	1	806.160	4.137	0.126	0.037	-401.017
Nest.Year + LPDV + Age + LPDV*Age	6	806.753	4.730	0.094	0.027	-395.761
Nest.Year + REV + Age + REV*Age	6	807.042	5.020	0.081	0.024	-395.906
Nest.Year + Coinf	6	807.604	5.581	0.061	0.018	-396.187
Nest.Year + Coinf + Age	7	808.410	6.387	0.041	0.012	-395.405
Nest.Year + Coinf + Age + Coinf*Age	9	813.209	11.186	0.004	0.001	-395.354

Table A.13. Initial AIC model selection comparing univariate models to identify relevant non-pathogen variables affecting nest initiation during the second nesting attempt of female wild turkeys captured and monitored from 2018–2020 in Maine. Age was kept as the baseline model in the second AIC model selection step.

Model	<i>k</i>	AICc	ΔAICc	ModelLik	Weight	LL
Age	2	129.353	0.000	1.000	0.681	-60.753
Null	1	132.161	2.808	0.246	0.167	-63.652
Weight	2	132.688	3.335	0.189	0.128	-62.421
Nest.Year	3	136.057	6.705	0.035	0.024	-62.362

Table A.14. Second AIC model selection to identify pathogen variables affecting nest initiation during the second nesting attempt of female wild turkeys captured and monitored from 2018–2020 in Maine. All models contain age as an explanatory variable, following Table S13. Interaction between the 4-category coinfection variable and age could not be assessed due to small sample size resulting in category singularities. Variables determined to be significant upon interpretation of coefficients and their 95% confidence intervals in a model containing all supported variables ($<2 \Delta$ AICc) are italicized.

Model	<i>k</i>	AICc	ΔAICc	ModelLik	Weight	LL
<i>Age</i>	2	129.353	0.000	1.000	0.585	-60.753
Null	1	132.161	2.808	0.246	0.126	-63.652
Age + LPDV	3	132.485	3.132	0.209	0.122	-60.576
Age + LPDV + LPDV*Age	4	132.485	3.132	0.209	0.122	-60.576
Age + REV	3	132.554	3.201	0.202	0.118	-60.610
Age + REV + REV*Age	4	134.591	5.238	0.073	0.043	-59.568
Age + Coinf	5	139.010	9.658	0.008	0.005	-59.305

APPENDIX B: Chapter 4 Supplemental Material

Table B.1. Summarized sampled parameter values of 229 lymphoproliferative disease virus haplotypes from MRBAYES phylogenetic analysis using a 25% burn-in. Estimates presented with mean and variance of sampled values, the lower and upper boundaries of the 95% credibility interval, and the median of the sampled values. Wild turkeys were sampled in Maine 2017–2020.

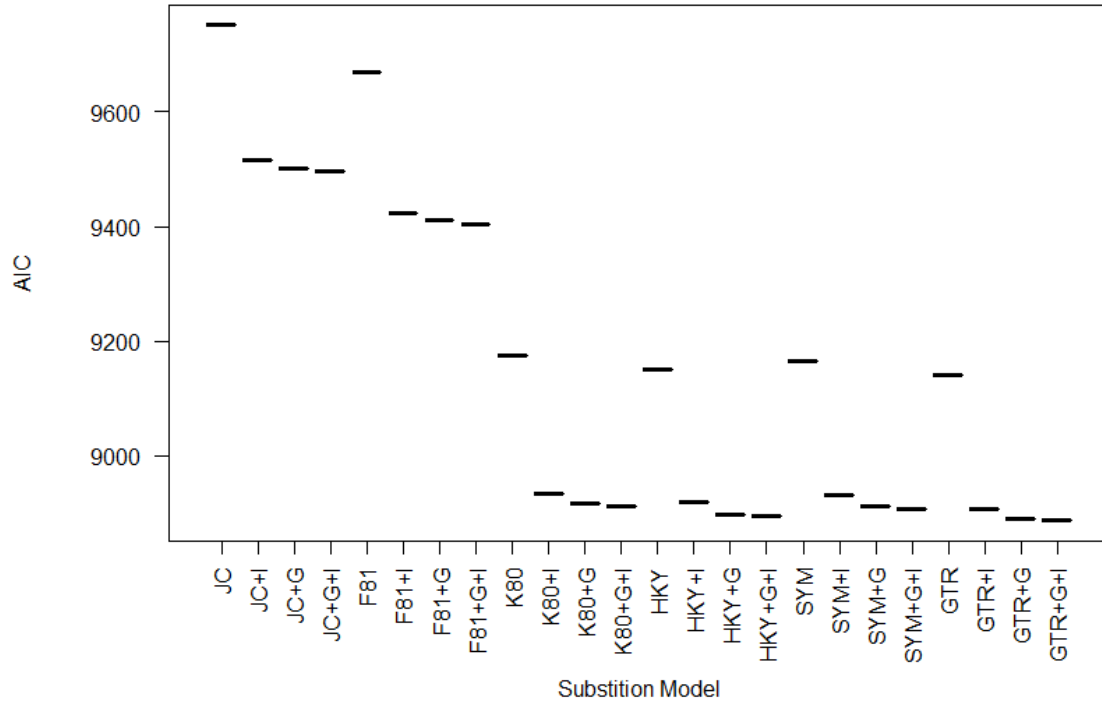
Parameter ^a	Mean	Variance	Lower	Upper	Median	minESS ^b	avgESS ^b	PSRF ^c
TL	1.134	0.0073	0.970	1.306	1.131	5084.440	5452.350	1.000
kappa	12.640	3.3631	9.195	16.252	12.488	10541.280	11199.900	1.000
pi(A)	0.275	0.0003	0.242	0.309	0.275	5415.310	6078.910	1.000
pi(C)	0.196	0.0002	0.166	0.226	0.196	7794.370	8517.370	1.000
pi(G)	0.321	0.0003	0.286	0.358	0.321	4460.300	6089.430	1.000
pi(T)	0.207	0.0002	0.178	0.238	0.207	4991.580	7272.250	1.000
alpha	0.461	0.0039	0.344	0.585	0.456	19710.400	20905.880	1.000

^aTL: total tree length (sum of all branches), Kappa: ratio of transition to transversion rates (r_{ti}/r_{tv}), Pi(A), (C), pi(G), pi(T): four stationary state frequencies, Alpha: shape of the gamma distribution of rate variation across sites.

^bminESS, avgESS: Effective Sample Size, the minimum and average value for the estimated effective number of independent draws from the posterior distribution that the MCMC sampled (Ripley 1987).

^cPSRF: Potential Scale Reduction Factor, comparison of the estimated between-chain variance with the within-chain variance for parameters (Gelman and Rubin 1992).

Figure B.1. AIC model selection of nucleotide substitution models of 229 lymphoproliferative disease haplotypes in 409 wild turkeys in Maine collected 2017–2020.



BIOGRAPHY OF THE AUTHOR

Stephanie Shea was born in Milford, Connecticut on December 4, 1989. She was raised in Orange, Connecticut and graduated from Amity Regional Highschool in Woodbridge, Connecticut, in 2008. She attended The University of Connecticut and graduated in August 2013 with a Bachelor of Science degree in Animal Science (Major; concentration: pre-veterinary studies) and Wildlife Conservation (Minor). She continued her education at Texas A&M University-Kingsville, particularly the Caesar Kleberg Wildlife Research Institute, where she received her Master of Science degree in Range and Wildlife Management in December 2016, researching helminth fauna in northern bobwhites. She returned to New England and entered the Ecology and Environmental Sciences graduate program at The University of Maine in January 2017. After receiving her degree, Stephanie will be joining The University of Maine Cooperative Extension as the Assistant Diagnostician in the Plant Disease Diagnostic Lab and The University of Maine Wildlife Disease Genetics Lab as a Postdoctoral Lab Manager and Research Scientist to continue her work as a wildlife disease ecologist. Stephanie is a candidate for the Doctor of Philosophy degree in Ecology and Environmental Sciences from The University of Maine in December 2021.