WEST NILE VIRUS EXPOSURE AND INFECTION RATES IN HUNTER-HARVESTED RUFFED GROUSE IN MINNESOTA

Charlotte Roy, Michelle Carstensen, Kelsie LaSharr, Carolin Humpal, and Ted Dick

SUMMARY OF FINDINGS

Minnesota participated in a collaborative, multi-state West Nile virus (WNV) study of ruffed grouse (*Bonasa umbellus*) in the Great Lakes region during 2018 and 2019. Cooperating hunters (n = 117) voluntarily collected 273 samples from birds harvested during the 2018 hunting season and hunters (n = 166) collected samples from 317 ruffed grouse during the 2019 season. We submitted blood on filter strips and hearts to the Southeastern Cooperative Wildlife Disease Study (SCWDS) at the University of Georgia to assess both 1) viral exposure as indicated by antibodies in blood, and 2) virus in hearts indicative of infection. Laboratory results indicated that 12.5% and 12.3% of birds were positive for antibodies to WNV or a flavivirus (most likely WNV rather than St Louis encephalitis virus, based on known WNV activity during the study period) in 2018 and 2019, respectively. However, virus was not isolated from hearts in either year, indicating that exposed birds were not infected at the time of collection. These findings indicate that Minnesota ruffed grouse are exposed to WNV. Some birds either mount a successful immune response without symptoms or develop symptoms and recover. However, any birds that might have succumbed to infection over the summer and did not survive were not available for sampling during the fall, so it is difficult to know how many birds might have been lost to WNV. Hunters and other citizens also submitted carcasses from 14 birds exhibiting abnormal behavior (e.g., unable to fly when flushed) or that had reduced pectoral muscle and a prominent keel. We submitted these presumably sick grouse to the University of Minnesota for necropsy and WNV screening. Eleven were also screened for Eastern Equine Encephalitis (EEE). All of these sick grouse were negative for WNV, but 4 were determined to be infected with EEE, which marked the first time this virus was directly linked to causing morbidity or mortality in a Minnesota wildlife species. Providing high quality ruffed grouse habitat that produces birds in good condition is our best management option for buffering ruffed grouse populations from WNV and other stressors.

INTRODUCTION

West Nile virus (WNV) is a mosquito-borne virus that infrequently causes a fatal neurological disease in people and horses, and can cause encephalitis and myocarditis in some infected birds. West Nile virus was first detected in a Ugandan woman in 1937 (Smithburn et al. 1940) and was first identified in birds, its natural reservoir host, in 1953 (World Health Organization, WHO, 2017). WNV was not considered pathogenic to birds until 1997 when a more virulent strain killed some birds in Israel. Today, avian mortality remains rare in Europe, Africa, and Asia, but mortality in birds is higher in the Americas (WHO 2017).

WNV was first detected in North America in 1999 when an outbreak of encephalitis was reported in humans in New York City (Eidson et al. 2001). Since its arrival in the U.S., WNV has become established in all of the lower 48 states and has been reported in over 300 bird species (Center for Disease Control 2017), including ruffed grouse. Although some species, like American crows (*Corvus brachyrhynchos*), blue jays (*Cyanocitta cristata*) and other Corvids, as

well as house sparrows (*Passer domesticus*) and common grackles (*Quiscalus quiscula*) readily die of WNV infection, most infected bird species survive (Komar et al. 2003). Interestingly, since the arrival of WNV to the U.S., mortality events due to WNV have never been documented in some bird species, (e.g. American robins (*Turdus migratorius*), chickadees (*Poecile spp.*), house wrens (*Troglodytes aedon*)), whereas other species had an initial period of reduced survival for several years until they gained immunity to the virus, and some species still continue to die from WNV annually (LaDeau et al. 2007, George et al. 2015).

The first documented WNV case in ruffed grouse in Minnesota occurred in 2005 (Ruffed Grouse Society, unpublished data). As many as 66 mosquito species have tested positive for WNV (Center for Disease Control 2019), but the suspected mosquito vector for ruffed grouse in Minnesota is *Culex restuans,* which feeds almost exclusively on birds and is abundant in forests (D. Neitzel, Minnesota Department of Health, personal communication). We suspect that ruffed grouse in northern Minnesota are exposed to WNV annually but do not know if the virus causes active infections that produce symptoms in juvenile or adult birds in the wild. Experimental infection of captive ruffed grouse and sage grouse (*Centrocercus urophasianus*) with WNV indicated high susceptibility of these species to the disease (Naugle et al. 2004, Clark et al. 2006, Nemeth et al. 2017). Furthermore, captive chick survival in ruffed grouse was negatively affected by the virus (Nemeth et al. 2017).

Concern for WNV in ruffed grouse in Minnesota was coincident with a publication on ruffed grouse and WNV from Pennsylvania (Stauffer et al. 2018) and heightened after the 2017 hunting season failed to meet harvest expectations. Hunters expected better-than-average hunting experiences, following a spring drumming count increase of 57% from the previous spring (Roy 2017), as has historically been the case when the 10-year cycle is nearing its peak (Amman and Ryel 1963, Stoll 1980). However, the drumming count is an index to the adult breeding population and these surveys occur before annual production, so drumming counts do not necessarily forecast the juvenile contribution to the fall population. Poor grouse production can adversely impact hunter experiences because juveniles comprise most of the fall harvest (Dorney and Kabat 1960, Dorney 1963). Despite 10-year cycles around a stable population average for decades in the core of Minnesota ruffed grouse range, some hunters have indicated that hunting experiences have been less rewarding during recent peaks in the cycle, leading to speculation that something has been affecting juvenile production.

We examined WNV exposure and infection rates in Minnesota ruffed grouse during the fall by partnering with grouse hunters to obtain samples from their harvested birds. We estimated serological exposure to WNV and also examined hearts for active virus infections. Juvenile birds may represent recent population exposure to WNV, for a direct correlation to current viral load on the landscape; whereas adult birds may represent either recent or maintained exposure, given the magnitude of their titer levels and presence or absence of virus.

Importantly, this study is a multi-state collaborative effort with other natural resource agencies in the Great Lakes Region, including Wisconsin and Michigan. This concerted effort will provide a more comprehensive view of the role of WNV in the region than any individual state could execute alone and demonstrates the interest of regional biologists in responding to hunter concerns.

OBJECTIVES

1. Assess the feasibility of working with grouse hunters to obtain biological samples from wild ruffed grouse for disease screening and to collect relevant metadata.
2. Estimate exposure to WNV in the fall ruffed grouse population by age class (juvenile and adult).

3. Determine prevalence of active infections (those producing symptoms or where the virus is reproducing) of WNV in fall ruffed grouse populations.
4. Correlate exposure to WNV with active infection using paired samples from the same bird.

METHODS

In 2018, our study area focused on a 60-mile radius around Grand Rapids, Longville, and Bemidji, MN. We chose this area in an attempt to sample along a moisture gradient from west to east, based on rainfall received the previous year, and to simplify logistics of sampling kit dissemination to a few pick-up/drop-off locations. Regional Minnesota Department of Natural Resources (MNDNR) headquarters are located in Bemidji and Grand Rapids and provided a location for distribution of sampling kits. Pineridge Grouse Camp, which is located in Longville, was committed to assisting with our sampling effort and provided a third location for distribution of kits. Numerous organized hunts were also conducted each year in the study area (e.g., Ruffed Grouse Society National Hunt, Northwoods Bird Dogs/Bowen Lake Lodge, Akeley Grouse Hunt), which further facilitated kit distribution and sample collection.

In 2019, we broadened our focal study area to include all of ruffed grouse range within Minnesota, to allow for participation by more hunters and provide a more dispersed sampling distribution within the forested part of the state. We distributed WNV kits to MNDNR Area Wildlife Offices throughout ruffed grouse range, based on anticipated demand by Area Wildlife Managers. As in 2018, we continued to make kits available at the regional offices in Grand Rapids and Bemidji and at Pineridge Grouse Camp in Longville, and worked with organized hunts. To further incentivize hunter participation, a shotgun (16Ga Stevens) and guided grouse hunt were offered as raffle prizes by the Ruffed Grouse Society and Pineridge Grouse Camp, respectively.

Hunter Outreach

We shared multiple press releases with the public with the first on 21 May 2018 announcing the multi-state collaboration between Wisconsin, Michigan, and Minnesota. Additional press releases on 23 Aug 2018 and 19 Aug 2019 provided more details for hunters interested in voluntarily participating in sampling efforts. Multiple media outlets shared progress about the sampling efforts throughout the hunting season to encourage public engagement (e.g., Duluth News Tribune, Outdoor News). The first year we gave presentations at local universities (e.g., Itasca Community College, Bemidji State University), hunting camps (e.g., Pineridge Grouse Camp), regional DNR staff meetings, and distributed kits during these visits. We also attended organized hunts to distribute and collect kits (e.g., Akeley Grouse Hunt, Ruffed Grouse Society National Hunt, Northwoods Bird Dogs/Bowen Lake Lodge). We added a short paragraph about the study to the 2018 and 2019 Minnesota Hunting Regulations and provided a contact for more information. Information about the study was available on the MNDNR website via the DNR Grouse Hunting Page. In fall 2019, we sent every hunter that participated in the 2018 surveillance a letter detailing the test results from their bird(s). We also had a multi-state press release on 22 Oct 2019 announcing the findings from the first season. We will mail letters with results for the fall 2019 sampling efforts to participating hunters in summer 2020.

Field Sample Collection

Each WNV sampling kit contained the following: 1 Nuboto filter strip (Advantec) for blood collection, 1 snack-sized zipper-top plastic bag (e.g., Ziploc brand) for storage of the filter strip in the field, a 3-inch coin envelope for storage of filter strip once blood had dried, a 4-oz whirlpak to collect the heart, a quart-sized zippered plastic bag for collection of feathers to confirm sex and age, and a datasheet to record hunter contact information and sampling location. In 2018, we also provided *A Grouse in the Hand* pamphlets with kits, courtesy of the Ruffed Grouse

Society. We provided a protocol in each sampling kit with detailed directions on how to determine the sex and age of harvested birds based on feather characteristics. The sex of ruffed grouse can be determined through tail length and rump feather dot patterns. Likewise, juvenile (<1 year) and adult (>1 year) age classes can be determined via feather wear of primary feathers collected from the wing in the fall. The instructions stressed the importance of collecting the blood on the filter strip within 30 min of harvest but also indicated samples collected after 30 min had value. We instructed hunters to thoroughly coat the filter strip with blood until uniformly red and to allow the strip to air dry following the hunt. We asked hunters to record date and time of harvest and blood collection, location of harvest (GPS coordinates or distance and direction from nearest town), county of collection, hunter-determined-age class (juvenile, adult, or unsure) and sex (male, female, or unsure), any relevant comments, and hunter contact information (address, phone, and/or email address) if communication of results was desired. We stored samples collected through organized hunts or through local hunting camps either at room temperature (feathers, Nuboto strips in 2018) or frozen (Nuboto strips in 2019, heart samples) until submitted. Otherwise, we provided hunters with mailing kits with pre-paid United Parcel Service shipping labels, along with freezer packs and thermal bubble mailers to keep samples cold during shipment the following business day. We confirmed age and sex of harvested birds before sending blood and heart samples to the Southeast Cooperative Wildlife Disease Study (SCWDS) at the University of Georgia (Athens, GA) for diagnostic testing after the end of hunting season.

Hunters or members of the public that encountered presumably sick grouse or discovered recently deceased birds occasionally reported these cases to MNDNR because of the ongoing study. When possible, carcasses from these birds were collected by MNDNR staff and stored frozen until submission to the University of Minnesota, Veterinary Diagnostic Laboratory (St. Paul, MN). Board certified pathologists performed whole necropsies to look for histological lesions consistent with clinical infection with WNV or Eastern Equine Encephalitis (EEE). Samples of brain and heart where outsourced to Cornell University to screen for WNV and EEE by PCR.

Laboratory and Data Analysis

Nuboto strips were reconstituted at SCWDS to test for antibodies to WNV using virus neutralization. Virus isolation was used to detect WNV and EEE in hearts. PCR was used to confirm virus presence. When virus was detected, histological examination of the tissue was performed.

We calculated apparent prevalence rates of WNV antibodies from serum collected with Nuboto strips using the number of positive detections relative to the total number of blood samples collected. We calculated seroprevalence for juveniles and adults separately. We calculated active infection rates using the number of PCR-positive tests of heart tissue divided by the total number of heart samples submitted. Both seroprevalance and active infection data were mapped using harvest location information.

We performed a space-time scan model in SaTScan v 9.6 (Kulldorff 2018; SatScan) to look for clustering of positive test results in space and time. We used a Bernoulli cluster**-**scanning model with positive and negative results to look for clusters of cases where relative risk of infection exceeded that in the surrounding area.

RESULTS

During 15 Sep 2018 – 1 Jan 2019, 117 hunters collected 273 samples from ruffed grouse harvested during the hunting season, of which 213 were collected from within the 60-mile sampling foci (Figure 1). Most of the returned kits contained all components requested, but 22

samples did not contain hearts, 40 samples were missing some or all feathers for sex or age determination, and 4 samples were missing location information.

During 14 Sep – 22 Dec 2019, 166 hunters collected samples from 317 ruffed grouse throughout the forested region in Minnesota (Figure 1). A road-killed grouse was submitted on 4 Jan 2020. Samples from one spruce grouse were also submitted, but excluded from analyses. Most submissions were complete, but 9 kits lacked hearts, 42 lacked the appropriate feathers for age determination, 10 lacked appropriate feathers for sex determination, and 5 were missing location information.

Antibodies consistent with WNV exposure, as indicated by WNV and flavivirus positive samples (i.e., most likely WNV, based on known WNV activity during the study, but possibly St. Louis encephalitis virus), were detected in 12.5% and 12.3% of samples in 2018 and 2019, respectively. Ten samples in 2018 and 3 samples in 2019 tested positive for WNV, with 24 and 36 positive for flavivirus in 2018 and 2019, respectively. None of the heart samples tested positive for WNV virus in either year. However, virus isolation resulted in two positive heart samples, but further testing by PCR confirmed Highland's J Virus, which is similar to Western Equine Encephalitis virus. Highland's J virus has a known distribution in the eastern USA and this was considered an incidental finding. Prevalence in Sep was similar to Oct in both years (12.9% and 11.3% in 2018, and 10.0% and 14.5% in 2019). Sample sizes were not sufficiently large in other months to calculate reliable prevalence estimates (range: 1 – 19).

Of the 14 carcasses of birds suspected to be sick and submitted for necropsy and testing due to abnormal behavior (e.g., unable to fly when flushed) or reduced pectoral muscle and a prominent keel, all birds were negative for WNV. Two of these birds were included in the tally of 317 hunter-submitted samples. Eleven were also screened for EEE, and 4 were determined to be infected with EEE.

Cohort Composition and WNV Prevalence

In 2018, we corrected the hunter-determined age in 50 of 211 cases (24%), and corrected sex 15 of 245 times (6%), not including cases where hunters indicated that they were unsure, or when feathers were not provided for verification. In 2019, we corrected the hunter-determined age 53 of 186 times (28%) and corrected sex in 24 out of 229 cases (10%).

In the sample of birds for which feathers were submitted to verify sex and age, the sex of sampled birds in 2018 (n = 212) was fairly evenly split between males (53%) and females (47%). In 2019, the sex ratio was more skewed in favor of males (64%) with 36% females (n = 235). The age of birds in the verified sample in 2018 was 64% juveniles and 36% adults; and the age ratio in 2019 was slightly more dominated by juveniles, 71% vs 29% adults. We used these samples to examine the prevalence among sex and age cohorts.

We determined the prevalence of WNV and flavivirus antibodies among verified age and sex cohorts for both years combined because sample sizes were fairly small for some cohorts when split among years (range: 21 – 102). Adults had similar prevalence to juveniles (15.1% vs 11.6%, Chi-square = 1.05, P = 0.31), and males had similar prevalence to females (14.9% vs 9.7%, Chi-square= 2.59, P = 0.11). Adult females (10.7%, n = 56) had similar antibody prevalence to juvenile females (10.1%, n = 129) and juvenile males (13.3%, n = 172), with adult males having the highest antibody prevalence though not statistically different (17.8%, n = 90, Chi-square = 3.08, P = 0.38). However, the power to detect statistical differences was low, as 650 individuals per cohort would be necessary to detect a 5% difference in prevalence.

Our spatial analysis did not reveal any significant statistical patterns among test results. We found a marginally significant cluster of positive cases about 20 km northeast of Hibbing, MN (P = 0.07).

DISCUSSION

A small but consistent proportion of hunter-harvested ruffed grouse in Minnesota tested positive for WNV and flavivirus antibodies from blood samples, indicating they had been exposed to virus, but we did not isolate virus in any of the submitted heart tissues. Assuming that false negatives were not an issue, which is a reasonable assumption given the short time for seroconversion to occur, at least some of the birds that were infected with WNV over the summer survived to the fall. We cannot know how many birds might have become infected with WNV and were either asymptomatic, experienced mild disease, or died from the virus over the summer.

Adult males had the highest antibody prevalence of all cohorts, and although not statistically higher, males had 1.66x the prevalence of females, or 7 percentage points higher than females, which is likely biologically relevant. Males might have higher prevalence because they utilize different summer areas than females and their broods (Mangelinckx et al. 2018), and they might experience higher exposure rates to mosquitoes carrying WNV in these areas. Males tend to utilize areas with greater densities of woody stems and less *Rubus* ground coverage than females with broods in the summer in Maine (Mangelinckx et al. 2018). However, we do not know if similar differences in summer habitat use occur in Minnesota, or how mosquito populations and exposure rates might vary across habitats in Minnesota, so sources of variation in exposure rates are not clear. Alternatively, once exposed to WNV, females may have higher mortality rates from the virus than males due to reproductive costs leaving females in poorer condition to face immune challenges and other stressors. Female ruffed grouse with broods had lower survival (69%) than those without broods and also males (98%) in Maine (Mangelinckx et al. 2018), and these findings are consistent with male-dominated sex ratios among harvested birds in our study and others (Dorney 1963, Davis and Stoll 1973). Adult sex ratios for hunter-harvested birds usually favor males, but juvenile sex ratios are usually closer to 50:50 (Dorney 1963, Davis and Stoll 1973). However, the juvenile sample in our study was also skewed towards males, although not as skewed as the adult sample (i.e., 43% juvenile female and 38% adult female). This skew among juvenile females might suggest that females are more susceptible to mortality from WNV or other causes and were less available for harvest in the fall by hunters, but this would require additional research to confirm and is speculative at this point. Juvenile females did have lower antibody prevalence than juvenile males, although not statistically significantly lower, as was seen in adults. However, sex differences in survival to WNV were not observed in American crows, a highly susceptible species to WNV, based on gender determination by discriminant functions (Yaremych et al. 2004). Male vertebrates often have higher disease incidence because testosterone is immunosuppressive (Grossman 1985), but higher antibody prevalence in male ruffed grouse is not consistent with immune suppression.

We found that females had similar antibody prevalence as juvenile birds. We would expect exposure of females and their broods to be similar over the summer because they spend so much time together. However, adult females might be expected to have higher prevalence of antibodies than their broods if WNV antibodies are maintained for an extended period and exposure occurred before broods hatched. The similarity in prevalence between juveniles, who could only be recently exposed to WNV, and adults could indicate that antibody titers do not persist between years for adults. Further study into WNV antibody persistence would be needed to provide a better understanding of titers in adults.

Prevalence of WNV and flavivirus antibodies in Minnesota ruffed grouse was similar or lower than prevalence found in other states, including Pennsylvania, where researchers conducted statewide serosurveys for WNV from hunter-harvested birds in 2016 and 2017 and found apparent prevalence rates of 14% (n = 202) and 22% (n = 217), respectively (J. Brown,

Pennsylvania Game Commission, unpublished data). In the Great Lakes region, Wisconsin reported a prevalence of 29% in 235 ruffed grouse samples and Michigan reported a prevalence of 13% in 213 samples in 2018. Data from our collaborating Great Lakes States during the 2019 sampling season were not available at the writing of this report. Unlike Minnesota, both Wisconsin and Michigan reported cases where WNV was detected in grouse hearts (2 and 4, respectively) in 2018. We cannot know if lower prevalence of antibodies in Minnesota is due to lower exposure rates to WNV or lower survival rates of exposed ruffed grouse, but given the lack of detections of virus in grouse hearts in Minnesota and the high abundance of forested habitat in Minnesota relative to other parts of ruffed grouse range, we suspect that lower exposure rates may be responsible for lower antibody prevalence and the absence of virus in hearts in Minnesota.

The first confirmed case of WNV in wild ruffed grouse in Minnesota was in 2005 (RGS, unpublished data). The first confirmed case in Michigan was in 2017 (Michigan Department of Natural Resources (MIDNR) 2017); 2 grouse were found dead, and 3 hunter-harvested grouse were submitted for testing because they were malnourished and acting strangely. In all 5 cases, heart lesions caused by WNV were observed (MIDNR 2017). The first time WNV was confirmed in Wisconsin ruffed grouse was also during this study, 3 birds tested positive for WNV, with 2 also being co-infected with EEE (Wisconsin Department of Natural Resources, WIDNR, unpublished data). Of the 14 suspect ruffed grouse carcasses submitted for testing in Minnesota since 2017, WNV was not detected, but 4 grouse were confirmed to have EEE.

This is the first time EEE has been confirmed in Minnesota ruffed grouse, although EEE is native to North America. This virus has been present in ruffed grouse populations in the region for a long time, with 50% of road-killed ruffed grouse having antibodies to EEE in a 1957 Wisconsin study (Karstad et al. 1960). However, the first clinical case of EEE was detected in Wisconsin in 2018 (WIDNR 2019). Evidence of EEE in any wildlife species in Minnesota was first discovered in moose and wolves, both showing exposure to the virus but without any evidence of direct morbidity or mortality (Butler et al. 2013, Carstensen et al. 2017). Primary ranges for both moose and wolves directly overlap with ruffed grouse range in Minnesota as well. These first EEE-infected ruffed grouse cases in Minnesota were reportedly exhibiting clinical signs of illness, and we likely only detected these cases because hunters were aware of the WNV study and knew how to submit carcasses of birds. Interestingly, these 4 birds all had lesions in the brain consistent with EEE infection and had no apparent heart lesions from the virus. Thus, it is possible that by relying only on heart tissue submissions for evidence of viral infection of WNV or EEE, we may be missing cases that have brain lesions, as brain samples were not part of our hunter-harvested surveillance. It is reasonable to assume that clinical cases of EEE occur each year in grouse, but go unreported.

Viruses have challenged wildlife populations for longer than wildlife managers have been managing wildlife. However, viruses become a concern when a population is naïve and newly exposed, or when other stressors impact the resistance of a population to immune challenges. For example, a recent study in Pennsylvania found a relationship between ruffed grouse population recovery in areas with poorer habitat and WNV (Stauffer et al. 2018). The interaction between invasive diseases and land-use can result in complex effects on survival of wild birds (George et al. 2015). Minnesota has a lot of forested habitat in a variety of successional stages that benefit grouse. Providing high quality habitat for grouse is our best option for buffering ruffed grouse populations against WNV because birds in good condition have stronger immune responses. We cannot currently reduce mosquito populations at a broad-scale or eliminate viruses, but we can try to provide the best habitat possible to produce grouse populations that are robust to a variety of stressors, including viruses.

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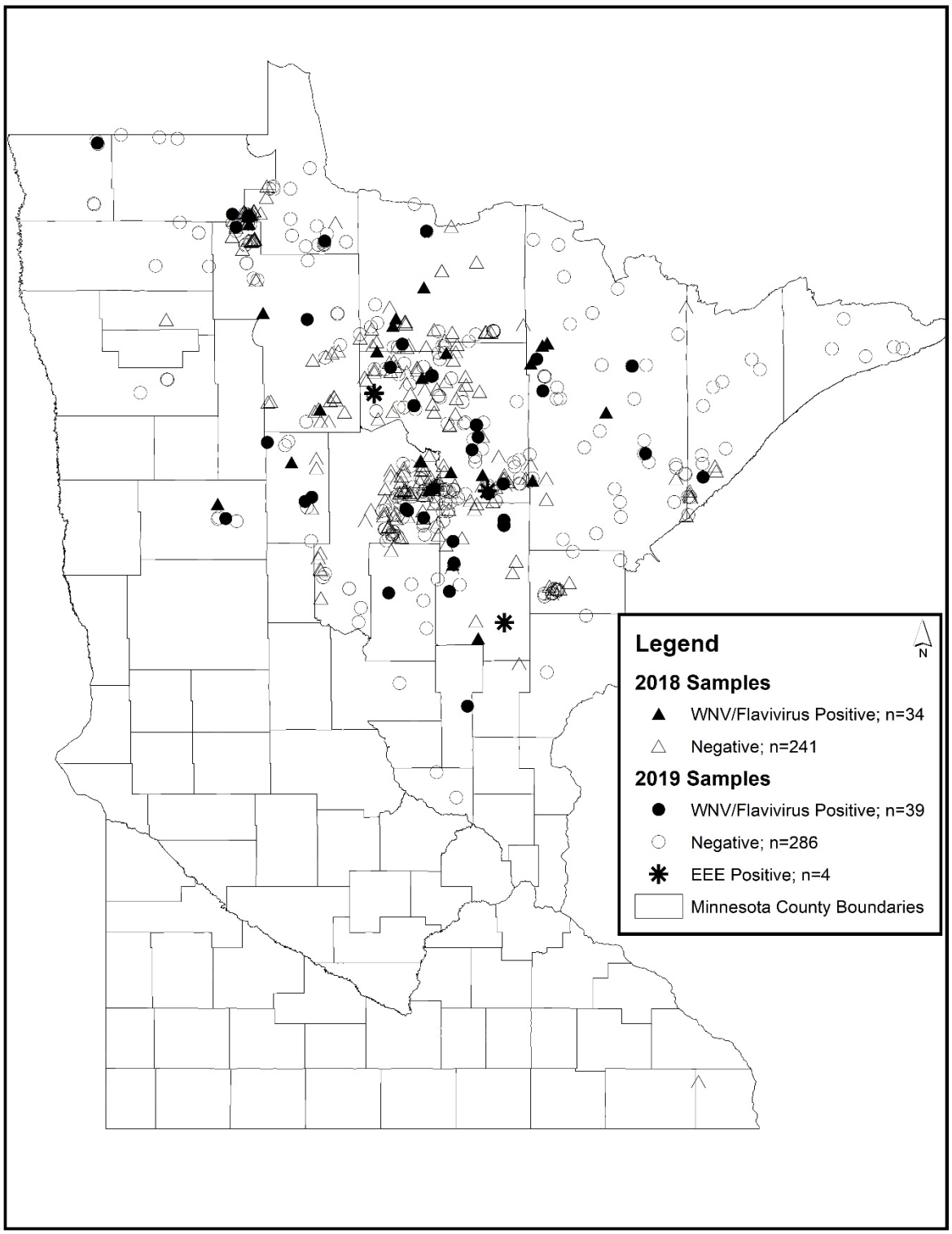

Figure 1. The distribution of hunter-harvested ruffed grouse samples and carcasses of presumably sick birds submitted for testing in Minnesota during 2018 and 2019. Samples are indicated as West Nile virus (WNV) positive/flavivirus positive (WNV suspect), negative, or Eastern equine encephalitis (EEE) positive.