

Experimental infection in the red-legged partridge (*Alectoris rufa*) with a North American West Nile virus strain

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Abstract

After the introduction of West Nile virus (WNV) into New York in 1999, bird mortalities associated with West Nile disease (WND) have dramatically increased in North America and, to a lesser extent, in Europe. The different WND incidence in birds in these two continents demands an explanation and experimental studies can provide important information. We inoculated thirteen 9-week-old red-legged partridges (*Alectoris rufa*) with 10^7 plaque forming units of a WNV strain isolated in New York in 1999. The objective was to study the pathogenesis of the infection in a native Euro-Mediterranean bird species with a WNV strain known to be highly pathogenic for numerous native American bird species. Additionally, we evaluated the dynamics of inflammatory cell activation and recruitment into the brain. The virus was detected in tissues as early as 3 days post inoculation (dpi) and the birds developed macroscopic and microscopic lesions. Two of the partridges succumbed to the disease. The most affected tissues were the heart, brain and spinal cord. The main microscopic findings were the presence of mononuclear infiltrates in heart and brain, gliosis and degeneration and necrosis of cardiomyocytes and neurons. These lesions were aggravated in the birds that died or were euthanized 7 dpi or later. In the brain, there was an up-regulation of microglial cells and astrocytes as well as an increase in the number of T cells, especially after 7 dpi. These results show that this WNV strain is of moderate virulence for the red-legged partridge, and that WNV-infected red-legged partridges develop an immune cell response in the brain similar to mammals.

Keywords: encephalitis, experimental infection, flavivirus, histopathology, immunohistochemistry, pathogenesis, red-legged partridge *Alectoris rufa*, West Nile virus.

West Nile virus (WNV) is a globally distributed mosquito-borne flavivirus whose main reservoir are birds.¹⁴ Before the 90's this arbovirus was considered a pathogen of limited importance for birds.³⁵ While West Nile disease (WND) has a high impact with significant mortality on bird species in the North American Continent,¹⁵ outbreaks in Europe are sporadic and have no impact on the population of the affected species. Several hypotheses have been proposed in order to explain this different eco-epidemiology such as different virulence of circulating strains or different susceptibility of Palearctic and Nearctic bird species to WNV infection.¹

Experimental infections of wild birds can greatly enhance the understanding of the eco-epidemiology and virulence of different WNV strains in its natural host.²⁴ In fact, susceptibility to both New and Old World WNV strains has been evaluated in different bird species.²⁴ However, this information is unbalanced as there are few experimental studies done in birds native to Europe while American species are fairly well represented.^{8,10,17,28} The study carried out in the red-legged partridge (*Alectoris rufa*) by Sotelo et al²⁸ constitutes the first experimental infection using a native European bird species. In this study, up to 70% of the birds developed disease and mortality after infection with two different Mediterranean WNV strains. In a different experiment, we demonstrated that the same species is also susceptible to a WNV strain isolated in North America, specifically in New York in 1999. This strain has been widely studied and is known to be highly pathogenic for certain native American bird species. In the aforementioned experiment, this WNV strain caused a mortality rate ranging from 14.3% to 45.5% (depending on the age at the time of infection).¹⁰ However, details addressing pathogenesis of the infection were not provided. For this reason, using tissues collected during the referred experiment, we describe here the

dynamics of virus appearance and distribution, as well as of macroscopic and microscopic lesion distribution and severity. Moreover, we studied the dynamics of inflammatory cell activation and recruitment into the brain. Neuropathogenesis of WNV infection has been thoroughly studied in rodent models in which it has been demonstrated that after virus entrance in the central nervous system (CNS), there is an activation of resident immune cells, production of immune mediators, and recruitment of peripheral inflammatory cells.^{2,6,7} The immune response in the brain of birds with WNV infection has not been assessed so far in detail in previous studies, although experiments in laboratory rodents have highlighted its importance in the pathogenesis of the WNV-associated encephalitis.¹⁸ Thus, our objective was twofold; to evaluate how the red-legged partridge responds to WNV infection in the CNS and to determine if this species could be used as a model to study the development of encephalitis in avian hosts.

Material and Methods

Virus

In this study a cell culture-passaged WNV New York/1999 (NY99) strain (GenBank accession number KC407666.1) isolated from a flamingo was used.^{5,19}

Experimental infection

As described in Escribano-Romero et al.,¹⁰ recently hatched red-legged partridge chicks were raised in the experimental farm of the Instituto de Investigación en Recursos Cinegéticos (IREC) until they were eight weeks of age. At that time, they were transported to the biosafety level 3 (BSL-3) facilities of the Centro de Investigación en Sanidad Animal (CISA) of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA). There, the birds were given five days for acclimatization. Prior to the experiment, oropharyngeal/ cloacal swabs and blood of

all birds tested negative for WNV-specific RNA by RT-PCR and negative for WNV antibodies by commercial ELISA (ID Screen, West Nile Competition, IDVet, Montpellier, France), respectively. For the present study, thirteen 9-week-old partridges were subcutaneously inoculated in the cervical region with 10^7 plaque forming units (PFU)/individual of the WNV NY99 strain diluted in up to 0.1 ml Dulbecco's Minimum Essential Medium (DMEM) (supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin). Animals were observed daily for clinical signs or death. For the specific purpose of our study, we prescheduled euthanasia at days 3, 7 and 10 (postinoculation (dpi). Three birds were found dead during the experiment and the six remaining ones were euthanized at 14 dpi.). Euthanasia was performed by inhalation anesthesia with isoflurane (Isoba®vet, Intervet – Schering-Plough, Madrid, Spain) and intravenous injection of embutramide (T61®, Intervet – Schering-Plough, Madrid, Spain). All animals were handled in strict accordance with the guidelines of the European Community 86/609/CEE and the protocols were approved by the Committee on Ethics of animal experimentation of the INIA (permit number 2010-005). Food and water were provided *ad libitum* throughout the experiment.

Sample collection

Necropsies were performed on all dead and euthanized individuals. Samples of brain, heart, liver, spleen and kidney were collected into sterile polypropylene tubes and stored at -70° C until analysis by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Additional samples of brain, oral mucosa, thymus, trachea, lung, heart, liver, spleen, kidney, adrenal gland, testicle, ovary, small and large intestine, pancreas, cecal tonsils, bursa of Fabricius, spinal cord, pectoral muscle and skin with feather follicles were fixed in 10% neutral buffered

formalin. For this study, samples from the partridges found dead (n=3, Nos. 1, 3 and 5), the partridges euthanized at 3 (No. 2), 7 (No.4) and 10 (Nos. 6 and 7) dpi (n=4), and two of the partridges euthanized at 14 dpi (Nos. 8 and 9, those with higher level of viremia at 3 dpi (data not shown)) were subjected to histopathological and virological examination.

Virus RNA detection

WNV RNA was extracted using a commercial Kit (Speedtools RNA virus extraction kit, Biotools B&M Labs SA, Madrid, Spain) and quantified by qRT-PCR.¹⁶ For RNA quantification a standard curve was generated with previously titrated WNV (10^6 – 10^{-1} PFU) and samples were considered negative when $Ct \geq 37$, equivalent to 9 PFU/sample (10^3 PFU/ml).¹⁰

Histopathology

Formalin-fixed tissue samples were trimmed, embedded in paraffin and processed to obtain 4 μ m sections that were stained with hematoxylin and eosin. These were independently examined by two different investigators (UH and VG) to determine the presence of WNV infection associated lesions. When lesions were present, these were graded according to their distribution (focal, multifocal or diffuse) and severity (mild, moderate or marked).

Immunohistochemistry

Tissue sections were also mounted on Vectabond™ reagent (Vector Laboratories, Inc., Burlingame, CA) pretreated slides for immunohistochemistry (IHC). Details of antibodies and reagents used for WNV antigen detection and inflammatory cell characterization in the brain are given in Table 1. Endogenous peroxidase activity was inhibited with a peroxidase blocking reagent (Dako EnVision®+System-HRP (AEC), DakoCytomation, Carpinteria, CA) (CD3, GFAP) or with 3% H₂O₂ diluted in

methanol (WNV antigen, RCA-1), slides were rinsed using 0.1% Tris-buffered saline/Tween20 (TBS 0.05 M, pH 7.5), non-specific primary antibody labeling was blocked with 2% albumin from bovine serum (BSA) (Sigma-Aldrich Chemie, Steinheim, Germany) diluted in 0.1% TBS/Tween20, and sections were counterstained with Mayer's hematoxylin. WNV antigen detection was carried out as described recently.¹¹

Tissue sections of red-legged partridges positive for WNV by RT-PCR served as positive controls. Negative controls included substitution of the primary antibody by 2% BSA-0.1% TBS/Tween20 and a negative rabbit antibody (BioReliance, Product 81-015, Rockville, MD, USA), as well as tissue sections of a non-infected (WNV RT-PCR negative) red-legged partridge. Sections of spleen of the same species served as IHC positive controls for T cells. In this case, negative controls included substitution of the primary antibody by 2% BSA-0.1% TBS/Tween20 and a brain section of a WNV RT-PCR negative partridge. A brain section of a non-infected partridge of the same age served as reference for RCA-1 and GFAP. Virus antigen staining was graded under the light microscope according to its distribution and abundance. Moreover, we studied the distribution of inflammatory cells within the brain parenchyma at a magnification of 200x. We counted the number of CD3+ T cells and RCA-1+ microglial cells/macrophages in 30 randomly selected fields (in each brain region: cerebrum, cerebellum, optic lobe, and pons and medulla oblongata) at different dpi at a magnification of 400x. Changes in the morphology, distribution and relative abundance of GFAP+ astrocytes during the course of the infection were also evaluated.

Results

Morbidity and mortality

The three partridges that died at 2 (No. 1), 7 (No. 3) and 8 dpi (No. 5) showed depression, ruffled feathers and recumbency starting 24 hours before death.

Macroscopic lesions

Macroscopic lesions were detected in every necropsied partridge. Differences between birds that died or were euthanized were minimal, with the exception of cachexia observed only in fatally infected birds and characterized by the absence of fat deposits and atrophy of the pectoral muscles. Lesions were more widespread between 7 and 10 dpi and the most affected organs were heart, spleen, liver and kidney. The most commonly observed lesions were diffuse pallor of the myocardium (Nos. 3, 6 and 7) and of the hepatic (Nos. 4, 8 and 9), splenic (Nos. 2, 5 and 9) and renal parenchyma (Nos. 1, 5 and 6), as well as splenomegaly (Nos. 2 and 4). In the liver, the partridge found dead at 8 dpi (No. 5) had multifocal irregular reddish areas and one of the birds euthanized at 10 dpi (No. 6) had multifocal pinpoint yellowish foci.

Other macroscopic lesions such as dilation of intestinal loops (7/9) and congestion of serosal vessels (4/9) (mainly in duodenum and cecum), as well as distension of crop with a whitish pseudomembrane covering the mucosa (2/9), were observed.

Virus RNA detection

The heart was the first tissue in which viral RNA was detected by qRT-PCR (3 dpi, No. 2) (Table 2). The kidney and spleen were positive from 7 and 10 dpi (Nos. 3, 6 and 7), respectively, but we did not detect viral RNA in the brain until 10 dpi (Nos. 6 and 7). At 14 dpi every organ was negative (Table 2).

Histopathology

Microscopic lesions were observed early in the infection, but their severity increased after 7 dpi (see supplemental material). The most severely affected tissues were the

brain, spinal cord and heart. In fatally infected animals, spleen, liver, kidney, large intestine, pancreas and bursa of Fabricius also showed moderate lesions. There were no microscopic findings in the trachea, proventriculus, cecal tonsils, testicles, ovaries, adrenal gland, and skin and feather follicles.

Main microscopic findings were the presence of inflammatory infiltrates, cellular degeneration and necrosis. While fatally infected animals showed moderate to marked lymphoid cell depletion and necrosis in lymphoid organs (characterized by karyorrhexis of lymphoid cells, accumulation of necrotic debris, and deposition of fibrin) (Nos. 3 and 5), this was rarely observed in the euthanized ones. In the heart, there were multifocal shrinkage and hypereosinophilia of cardiomyocytes, with occasional loss of cross striation, and pyknosis or karyorrhexis of the nucleus (Fig. 1). Mild edema and multifocal inflammatory infiltrates composed of lymphocytes, plasma cells and macrophages were also observed (Fig. 1). The lesions were more severe in the animals that died between 7 and 10 dpi, particularly in fatally infected animals (Nos. 3 and 5). Massive hepatic necrosis characterized by individualized, shrunken, rounded, hypereosinophilic hepatocytes with pyknotic or karyorrhectic nuclei, was observed in the partridge found dead at 7 dpi (Fig. 2). The partridge euthanized at 7 dpi (No. 4) had multifocal random areas of hepatocyte necrosis and deposition of fibrin as well as multifocal infiltration of small numbers of lymphocytes, plasma cells, macrophages and heterophils, mainly in the sinusoids. Scattered Kupffer cells with a brownish pigment in the cytoplasm (likely hemosiderin) were also detected. Mild hemosiderosis was a common feature, also in the spleen. As early as 3 dpi (partridge No. 2) tubular epithelial cells in the renal cortex were hypereosinophilic, shrunken, detached from the basement membrane and showed pyknosis and/or karyorrhexis of the nucleus. These changes were more severe in the

partridge found dead at 7 dpi (No. 3), in which the tubular lumen often contained eosinophilic granular to fibrillary material (protein casts). Multifocal lymphoplasmacytic and histiocytic interstitial nephritis was also observed in this bird and in one of the partridges euthanized at 10 dpi (No. 6). Multifocal infiltration of small numbers of lymphocytes in the septum of the air capillaries of the lung was observed in partridges No. 4 and 7 (euthanized at 7 and 10 dpi, respectively).. Multifocal infiltration of lymphocytes, plasma cells and macrophages were observed in the muscularis externa of the gizzard of the birds that died at 8 dpi (No. 5) and were euthanized at 10 dpi (Nos. 6 and 7), and in the lamina propria of partridge No. 7. Similar inflammatory cells were found in the muscularis externa of the duodenum and large intestine. Large numbers of heterophils were also present in the lamina propria of the duodenum of the partridges euthanized at 14 dpi (Nos. 8 and 9) and the large intestine of one of the partridges each euthanized at 10 and 14 dpi (No. 6 and 8, respectively). The two fatally infected partridges (Nos. 3 and 5) had necrosis of acinar pancreatic cells, characterized by individualized cells displaying shrinkage, hypereosinophilia, occasional loss of zymogen granules, and vacuolation of the cytoplasm as well as pyknosis and/or karyorrhexis of the nucleus and loss of polarity. In the CNS, a non-purulent encephalitis characterized by small to moderate numbers of lymphocytes, plasma cells and macrophages, mainly expanding the Virchow-Robin space (perivascular cuffing) was observed from 7 dpi on. Increased numbers of glial and microglial cells (gliosis) that often formed aggregates (glial nodules) were also detected (Fig. 3, supplemental material). In the cerebrum, optic lobe and spinal cord of fatally infected partridges (Nos. 3 and 5) and one of the partridges euthanized at 10 dpi (No. 7), there were scattered neurons with hypereosinophilic contracted cytoplasm and pyknotic, occasionally karyorrhectic, nuclei (degeneration and

necrosis). Mild to moderate Purkinje cell necrosis was also observed in these animals (Fig. 4). Additionally, multifocal neurons and Purkinje cells were surrounded by low numbers of glial cells (satellitosis), mainly between 8 and 10 dpi. Another common finding that appeared as early as 3 dpi was endothelial cell swelling. Incidental findings included intestinal coccidiosis in two fatally WNV-infected birds (Nos. 3 and 5) and crop mycosis in one fatally (No. 3) and five euthanized (Nos. 2 and 6-9) partridges.

Immunohistochemistry

Virus antigen detection

Although WNV antigen detected by IHC was relatively scarce, it was widespread, especially between 7 and 10 dpi (Table 3). Positive immunostaining was observed in the cytoplasm of cardiac myofibers (Fig. 5), glomerular mesangial and tubular epithelial cells of the kidney, and acinar cells of the pancreas as early as 3dpi (Table 3). In the spleen, WNV antigen was detected in macrophages (3 and 8 dpi) and in few smooth muscle cells of vascular walls (10 dpi). Only the two birds found dead at 7 and 8 dpi showed positive staining in hepatocytes and Kupffer cells and in proventricular gland epithelial cells. In one of the partridges euthanized at 10 dpi (No. 6), WNV antigen was detected in inflammatory cells in the lamina muscularis of the gizzard and in muscular fibers of the lamina muscularis of the large intestine. In the CNS, the two birds euthanized at 10 dpi showed positive staining within the cytoplasm of one Purkinje cell and very few dendrites as well as in scarce glial cells in the cerebellum (Fig. 6). At 14 dpi, viral antigen was only detected in case No. 8 in one cardiac myofiber (Table 3).

Inflammatory cell activation and recruitment into the brain

Inflammatory cell activation and recruitment into the brain were only studied in euthanized partridges.

a) Microglia activation/macrophage infiltration

Activated microglial cells labeled by their expression of RCA-1 were observed as early as 3 dpi. There were changes in their morphology, distribution and abundance during the course of the infection and among brain regions (Fig. 7). At 3 dpi, microglial cells displayed a ramified morphology and were found diffusely distributed within the parenchyma. From 7 dpi onwards, microglial cells changed to an activated amoeboid morphology (large soma and short thick cellular processes). Numerous microglial/macrophage nodules were present, in some areas surrounding necrotic cells. After 10 dpi microglia activation and macrophage infiltration slightly decreased, in the cerebrum, cerebellum and optic lobe, but not in the pons and the medulla oblongata (Fig. 7).

In the cerebrum RCA-1+ cells were more numerous in the peripheral pallium and intensely stained near the lateral ventricle. In the cerebellum, RCA-1+ cells were more numerous in the molecular layer (Fig. 8). In the optic lobe microglial nodules were mainly located in the *stratum griseum et fibrosum superficiale*. In the pons and medulla oblongata there were multiple microglial nodules at 14 dpi, some of them located in nuclei.

b) Astrocyte activation

Astrocytosis (demonstrated by an increase in the number of GFAP+ cells) was mild at 3 dpi. Between 3 and 10 dpi, no significant increase in number of positive cells was observed. At 14 dpi, astrocytosis was more prominent.

In the cerebrum, multifocal aggregates of GFAP positive astrocytes were detected in the peripheral pallium as early as 3 dpi. At this time, a mild increase in the length of

cellular processes in the astrocytes located in the subpial region was also observed. Numerous star-shaped astrocytes were stained near the lateral ventricle, especially in the two partridges euthanized at 14 dpi. In the cerebellum, astrocyte activation was observed as early as 3 dpi in the granular layer, but it was not until 14 dpi when the number of positive cells also increased in the white matter. In the optic lobe numerous star-shaped astrocytes stained GFAP positive in the *stratum opticum* (some of them surrounding blood vessels) and at 14 dpi a mild increase in the number of positive cells was observed in the *stratum album centrale*. In the pons and medulla oblongata there was a diffuse meshwork of GFAP+ cells. Multifocal aggregates of astrocytes were detected as early as 3 dpi but these were more numerous at 14 dpi.

c) T cell infiltration

CD3+ T cells infiltrated the brain after 7 dpi. Changes in their distribution and abundance during the course of the infection and differences among brain regions were observed (Fig. 9). T cells were found diffusely distributed but also forming part of inflammatory nodules (surrounding necrotic cells) and perivascular infiltrates. At 14 dpi, with the exception of the pons and medulla oblongata, cellular infiltration decreased markedly (Fig. 9). Similarly to RCA-1+ cells, T cells were especially numerous in the peripheral pallium of the cerebrum and in the molecular layer of the cerebellum. In the optic lobe, the *stratum opticum* and *stratum griseum et fibrosum superficiale* and, to a lesser extent, the *stratum griseum and album centrale* showed mild T cell infiltration. The pons and medulla oblongata were the more markedly infiltrated regions and showed numerous nodules (Fig. 10) corresponding in many cases to microglia activation areas.

Discussion

In this work we describe the pathogenesis of the experimental infection of juvenile red-legged partridges, a native Euro-Mediterranean gallinaceous bird species, with a North American WNV strain. To our knowledge this is one of the few experimental WNV infections carried out in a gallinaceous bird species. It is also one of the few studies in which lesions associated with WNV infection is thoroughly reviewed in a native European bird species, and one of the few characterizations of the inflammatory response in the CNS of WNV infected birds.

Most infected partridges developed multiorgan macroscopic and microscopic lesions which, as expected, were more severe and widespread in fatally infected birds and as infection progressed. The partridge that died as early as 2 dpi showed mild microscopic lesions and negative results in the WNV qRT-PCR and IHC. Considering this, the fact that the partridge euthanized at 3 dpi had mild acute lesions, and that even highly susceptible species (crows or jays) do not usually succumb to WNV infection until 4-5 dpi,^{23,32} we cannot rule out other underlying causes of death for this individual.

The vast majority of observed lesions were similar to those described in other birds naturally and experimentally-infected with WNV.^{22,29,37} The most severely affected tissues were the CNS and heart. Lesions in the CNS have also been described in WNV-infected gallinaceous birds such as chickens (*Gallus gallus*), Impeyan pheasants (*Lophophorus impeyanus*) and wild turkeys (*Meleagris gallopavo*).^{27,29,36} The heart is one of the main target organs in naturally and experimentally WNV-infected gallinaceous birds.^{27,29,30,36} In fact, in two different WNV outbreaks in chukar partridges (*Alectoris chukar*), a species closely-related to the red-legged partridge, myocarditis and myocardial necrosis were the main microscopic lesions and WNV antigen was detected in cardiomyocytes by IHC.^{9,34}

The susceptibility of the red-legged partridge to WNV infection had also been demonstrated by Sotelo et al²⁸ using two Mediterranean strains, Morocco/2003 and Spain/2007. Despite the lower dose inoculated (10^3 PFU) in that case, the virus intensely replicated in the brain, heart, liver and spleen and mortality rate was as high as 70% for Morocco/2003; however, differences in experimental conditions, analytic methods and the low number of birds tested do not allow us to compare the virulence of these strains for the same species.

Considering the high viral inoculation dose (10^7 PFU), virus detection in tissues by IHC and qRT-PCR was relatively low. Nevertheless, tissues with the most prominent microscopic lesions such as brain, heart, spleen and, to a lesser extent, kidney were those with positive results using both techniques. The presence of moderate microscopic lesions in tissues, such as the brain and heart, of birds euthanized at 14 dpi in absence of viral antigen and viral RNA may indicate elimination of the virus by the immune response of the host. The immune response may also contribute to the severity of lesions found in these tissues, as it has been previously suggested for other bird species.³ In rodent models it has been demonstrated that despite the importance of inflammatory cells for the elimination of the virus from the CNS, an exacerbated and prolonged activation or recruitment of inflammatory cells can have detrimental effects, contributing to neuron damage.^{2,31} The inflammatory response in the brain of our WNV-infected partridges was characterized by an early mild reaction of resident immune cells, though their number increased and morphology changed as infection progressed. Astrocyte activation was strongest at 14 dpi in the partridges of this study. This observation is in agreement with findings in mammals in which astrocyte activation is delayed compared to microglial activation and frequently peaks at 14 days post injury,²¹. Astrocytes and microglia also participate in the anti-flavivirus

response in mice and humans.^{2,12,13,20} In response to resident immune cell activation, and probably to other stimuli, CD3+T cells infiltrated the brain from 7 dpi onwards, being especially numerous at 10 dpi. It has been indicated that T lymphocytes are essential to destroy WNV-infected cells and for CNS recovery, both in humans and rodents.^{2,26} It is important to highlight that the pons and medulla oblongata were the brain regions with the most significant inflammatory reaction. Similarly, in humans and horses, these regions are the primary sites of lesions of WNV-associated encephalitis.^{4,25} In fact, naturally infected birds usually show microscopic lesions in this area.^{29,33,36}

Conclusion

In conclusion, the red-legged partridge, a native Euro-Mediterranean gallinaceous bird species, develops lesions and moderate mortality when infected with a North American NY99 WNV strain. The relatively low mortality observed, despite the high virus inoculation dose used (10^7 PFU/individual) indicates that this virus strain is of moderate virulence for this bird species. The study of the immune cell activation and recruitment into the brain demonstrates that this avian host responds to WNV infection similarly to experimentally-infected mammals. Thus this bird species could be a suitable model to study WND and the development of encephalitis in avian hosts.

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Conflicts of interest

The author(s) declared no potential conflicts of interest.

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Figures 1-6. Experimental West Nile virus (WNV) infection, red-legged partridges.

Figure 1. Heart; partridge No. 5, found dead 8 days post inoculation (dpi). Focally extensive myocardial degeneration and necrosis with infiltration by lymphocytes, plasma cells and histiocytes. Necrotic myofibers show lysis of the nucleus and contraction and hyalinization of the sarcoplasm. Hematoxylin and eosin (HE).

Figure 2. Liver; partridge No. 3, found dead 7 dpi. Massive hepatic necrosis, with individualized hepatocytes showing hypereosinophilic cytoplasm and nuclear pyknosis, and surrounded by cellular debris. HE.

Figure 3. Cerebrum; partridge No. 5, found dead 8 dpi. Diffuse moderate gliosis, lymphoplasmacytic and histiocytic perivascular infiltration and satellitosis (arrowheads). HE.

Figure 4. Cerebellum; partridge No. 7, euthanized 10 dpi. Glial nodules in the Purkinje cell layer and molecular layer. HE.

Figure 5. Heart; partridge No. 5, found dead 8 dpi. WNV antigen in the cytoplasm of cardiac myofibers. Immunohistochemistry (IHC) using a WNV rabbit polyclonal antibody.

Figure 6. Cerebellum; partridge No. 6, euthanized 10 dpi. WNV antigen in the cytoplasm and dendrites of Purkinje cells. IHC using a WNV rabbit polyclonal antibody.

Figures 7-10. Inflammatory cell recruitment and activation in encephalitis due to experimental West Nile virus (WNV) infection, red-legged partridges, brain.

Figure 7. Semi-quantitative analysis of RCA-1+ microglial cells/macrophages in different brain regions at different dpi. Bars represent the mean of stained cells in 30 randomly selected 400x fields \pm standard deviation. Control is a non-WNV infected partridge.

Figure 8. Cerebellum; partridge No. 7, euthanized 10 dpi. RCA-1 positive staining in the cytoplasm of activated microglia/macrophages forming nodules in the molecular layer. IHC for microglia activation/macrophages.

Figure 9. Semi-quantitative analysis of CD3+ T cells in different brain regions at different dpi. Bars represent the mean of stained cells in 30 randomly selected 400x fields \pm standard deviation.

Figure 10. Medulla oblongata; partridge No. 7, euthanized 10 dpi. CD3 positive staining in the cytoplasm of T cells forming an inflammatory nodule in the neuropil. IHC for T cells.