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Title: Synergized resmethrin and corticosterone alter the chicken's response to West Nile virus infection

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Running Head: Multiple stressors and chicken immunity to WNv

Keywords: insecticide; resmethrin; West Nile virus; stressor; corticosterone; avian immunology; mosquito control; public health; ecological health; chicken

Abbreviations and Definitions:
USGS: US Geological Survey
BRD: Biological Resources Discipline
NWHC: National Wildlife Health Center
USEPA: US Environmental Protection Agency
UW-Madison: University of Wisconsin-Madison
METC: Molecular and Environmental Toxicology Center
NYDEC: New York State Department of Environmental Conservation
NIEHS: National Institutes of Environmental Health Sciences
NRSA: National Research Service Award
BSL: Bio-safety level
WNv: West Nile virus
WNv_E: West Nile virus envelope protein
SR: synergized resmethrin
CORT: corticosterone
DPI: day(s) post inoculation
PFU: plaque-forming units
Adulticide: aerially applied synthetic insecticide used to kill adult flying mosquitoes

Abstract

Background: Wild birds are an important reservoir host for WNv and are potentially exposed to insecticides used for mosquito control. However, no risk assessments have evaluated whether insecticides augment or extend the potential transmissibility of WNv in birds.

Objective: In order to augment existing resmethrin risk assessments, we aimed to determine whether synergized resmethrin (SR) may cause chickens to develop an elevated or extended WN viremia and if subacute stress may affect its immunotoxicity.

Methods: We distributed 40 chickens into four groups then exposed them prior to and during WNv infection with SR (50 µg/l resmethrin + 150 µg/l piperonyl butoxide) and/or 20 mg/l corticosterone (CORT) in their drinking-water. Corticosterone was given for 10 continuous days and SR was given for 3 alternate days starting the 3rd day of CORT exposure, then chickens were subcutaneously inoculated with WNv on the 5th day of CORT treatment.

Results: Compared to controls, CORT treatment extended and elevated viremia, enhanced WNv-specific antibody and increased the percentage of birds that shed oral virus, whereas SR treatment extended viremia, depressed WNv-specific IgG, and increased the percentage of CORT-treated birds that shed oral virus

Conclusions: Corticosterone and SR independently and interactively altered immunity to WNv in chickens. Further characterization of how variations in SR-exposure to and CORT levels in chickens and wild birds relate to laboratory WNv-infection trials is warranted in order to place these findings into an epidemiological context.

Introduction

Debate concerning arbovirus control strategies remains contentious because concern regarding the relative risk of viral infection and environmental toxicant exposure is high but inadequately characterized. Taking this into account, mosquito control agencies employ aerial insecticides only after arbovirus surveillance data indicate high local mosquito-infection-rates. Successfully mitigating the risk of adult-mosquito-control insecticides ("adulticides") to non-target species such as humans, domestic animals, fish, beneficial insects and wildlife, while increasing their efficacy to reduce arbovirus outbreak intensity requires targeted scientific data from animal toxicity studies and environmental monitoring activities. For example, pyrethroid spraying activities were recently shown to reduce the number of flying mosquitoes (Elnaiem et al 2008), while a related study indicated, for the first time, fewer human West Nile virus (WNv) cases in sprayed compared to unsprayed areas (Carney et al 2008). Although these studies demonstrated short-term insecticide efficacy during specific outbreaks, spatiotemporally varied conditions such as vegetation density and mosquito resistance to a particular insecticide can affect mosquito-killing efficacy. Moreover, these studies did not and very few others have monitored environmental concentrations of adulticides or potential adulticide-exposure in non-target organisms in conjunction with mosquito control activities. Those that have investigated environmental concentrations of pyrethroids after adulticiding have detected resmethrin at 0-0.293 ppb (Abbene et al 2005), permethrin at 0-9.40 ppb (Pierce et al 2005) and piperonyl butoxide synergist at 0-60 ppb (Abbene et al 2005, Shleier et al 2008). Permethrin in sediments near agricultural

activities have been found up to 459 ppb (Weston et al 2004). Higher sediment than water pyrethroid concentrations are not surprising given the hydrophobicity of pyrethroids, suggesting that non-target species' oral exposures could be of a pulsed (water) or chronic (food) nature.

Evaluating the risk of insecticides to non-target species prior to governmental registration involves a consideration of the chemical's environmental fate and transport and any potential effects to those species given predicted environmental concentrations. However, risk assessment procedures often mandate only an evaluation of the active ingredient in a commercial formulation of an insecticide and do not require techniques that evaluate avian immunotoxicity (USEPA 2008). This is an important issue for insecticides used to control WNv or other arboviruses because an immunosuppressed avian host may be more infectious to mosquitoes. And, given the potential for elevated public health risk through incidental insecticide exposure of avian WNv hosts, a comprehensive evaluation of an adulticide's risk should take into account the effect of variation in host health status (e.g., short-term elevations of the "stress-hormone", corticosterone) on the immunotoxicity of a commercial formulation of an insecticide.

During arbovirus control efforts, the formulation of resmethrin that is disseminated includes the synergist piperonyl butoxide (PBO, a p450 inhibitor (Casida 1980)) and petroleum distillates. Resmethrin is highly toxic to terrestrial invertebrates, such as honey bees (*Apis mellifera*) (Murray 1985), and many aquatic taxa (Demoute 1989) including lobsters (Zulkosky et al 2005) and fish (Paul et al 2005), but is considered relatively benign to mammals and birds (Neuschl et al 1995, e.g.).

However, various investigators have found altered immune responses in mice, (Blaylock et al 1995) chickens (McCorkle et al 1980) and lobsters (De Guise et al 2005) exposed to pyrethroids while others have shown that permethrin activates the HPA axis in mice (de Boer et al 1988). These studies are insufficient for an assessment of the human (Peterson et al 2006) or ecological (Davis et al 2007) risks of adulticides because they used only the active ingredients of a commercial insecticide and exposed only healthy subjects, not recognizing that physiological status can vary greatly in nature (McEwen and Wingfield 2003). Thus, we performed this trial to determine if a WNv-resistant species, the domestic chicken (*Gallus domesticus domesticus*), might become more susceptible to WNv when exposed to 3 alternate days of ppb concentrations of synergized resmethrin (SR), and if subacute elevations of corticosterone influences its immunotoxicity. We characterized immunotoxicity by evaluating the epidemiologically important parameters of antibody response, oral and cloacal shedding of virus, and viremia. We show that corticosterone and ppb levels of SR suppressed immunity to WNv in different ways, and that corticosterone marginally affected SR's immunotoxicity. These data enhance our understanding of the chicken's immunity to WNv upon physiological stress, but complicate interpretations of the risk of resmethrin to avifauna and perhaps its public health utility.

Materials and Methods

Experimental Design

Forty-seven SPF chicken eggs were obtained from Charles River Laboratories (Chicago, IL). The eggs were hatched, and chicks were raised without handling for 6 weeks at the University of Wisconsin-Madison Poultry Research Laboratory (UW PRL), Madison, WI, and then moved to a BSL-3 facility (USGS NWHC, Madison, WI) for the remainder of the experiment. We randomly distributed 40 chickens to 4 groups of 10. These birds were maintained at a 12:12 light:dark cycle and fed *ad lib* with UW PRL ration.

Each group of 10 chickens drank water mixed with one of four possible combinations of SR and corticosterone (CORT). Two groups were exposed to 20 mg CORT / L drinking water for 10 days to simulate subacutely elevated adrenal activity and 2 groups were exposed to SR (50 μg resmethrin + 150 μg PBO + petroleum distillates/L drinking water; diluted from Scourge4+12®, Bayer Environmental Science, Research Triangle Park, NC) in a 0.10% ethanol vehicle. We based the CORT concentration on a pilot study in which immunity and corticosterone were measured (Jankowski, unpublished data), and on a study performed by Post et al (2003) that showed predictable immune suppression without affecting outward appearance and behavior. The SR water concentrations used in this study were determined by extrapolating from the manufacturer's label instructions (3 parts resmethrin: 1 part PBO at a rate of 3.18 g resmethrin/acre) if 1 acre of wetland was exposed and the upper 6 inches of water was sampled (Terracciano, personal communication). Chickens were

challenged subcutaneously with either 100 μl bovine-albumin viral media (BA-1) containing 10^5 PFUs of American crow isolate 16399-3 WNv or with 100 μl BA-1.

Sampling Protocol

After delivery from the brooder facility, the chickens were not handled for 7 days. On the 8th day of housing (-16 DPI), mock fecal sampling was performed daily for 7 days to allow the chickens to become familiar with the fecal sampling process. Each bird was captured from its pen and placed in a plastic poultry crate (0.142 m^3) within the larger pen until defecation occurred (~10 minutes). Due to the amount of time required to complete sampling (1-2 hrs/day) and the known circadian rhythm of CORT, we controlled for this by sampling a different group first each day.

Starting on -9 DPI, fecal samples (with minimal uric acid) were collected daily to establish a baseline CORT level prior to chemical and WNv treatments. Fecal samples were held on ice for up to 1 hour prior to freezing at -20°C. Blood samples, and oral and cloacal swabs were collected on -9 DPI for baseline virology and serology, and on DPI 1 – 5, 7, 10, and 14 to track the immune response to WNv. A blood smear was made, and blood samples were allowed to clot at room temperature for 30 minutes, chilled on wet ice, then centrifuged at 5000 x g for 15 minutes; serum was removed and frozen at -80°C until analysis. All oral and cloacal swabs were chilled on wet ice after collection, and then frozen at -80°C within 1 hour of sampling. Birds were weighed every blood-sampling day. All birds were euthanized via CO_2 asphyxiation on 14 DPI.

Analysis of Fecal Metabolites of Corticosterone

We measured metabolites of CORT (Möstl et al 2002) in fecal samples to relate a non-invasive measure of "stress" to viremia and to verify that CORT given in drinking water was absorbed across the gastrointestinal tract after ingestion, and biologically available. Samples were thawed from -20°C to 95°C for 30 minutes to inactivate potentially metabolite-altering bacterial enzymes. Samples were then weighed and suspended in 5 mls 60% methanol-water for each gram of wet feces. To liberate metabolites of CORT from the feces, samples were vortexed on a multitube vortexer for 30 minutes, followed by 20 minutes of centrifugation at 2000 x g to clarify the suspension. One ml of this suspension was aliquoted in a 2.0 ml cryovial, evaporated at 60°C for 24 hours and frozen at -20°C until analyzed for a metabolite of CORT in chickens (11-oxoetiocholanolone) by EIA according to Möstl et al 2002 and 2005. This assay reacts with metabolites of a 3-α-11-oxo structure.

White Blood Cell Differential Counts

Blood smears were stained with Wrights-Giemsa. The heterophil:lymphocyte ratio was calculated after identifying a total of 100 of these cells per smear at 1000X. This was performed as another measure of "stress" (Gross and Siegel 1983).

Serum Antibody (IgG and IgM) to WNv_E

Serum anti-WNv envelope protein (WNv_E)-IgG antibodies were detected by using a sandwich ELISA developed in our laboratory. We detected IgM using procedures based on Johnson et al (2002). See supplementary material for assay protocol details.

Virus Detection

Vero cells were used to detect the presence of virus in serum and oral swab media by plaque formation. Viremia (PFU/ml serum) was calculated from the serum dilution that produced between 1 and 30 plaques per well.

Statistical Analysis

Prior to and following appropriate data transformations, data were checked for distribution type (Gaussian or Poisson). All data were analyzed for main (SR and CORT) and interactive (SR*CORT) effects using a 2 X 2 model. Cell culture time-course data were analyzed with a Poisson-linked generalized linear mixed model in which treatment was the fixed effect and subject was the random effect ('lmer' function, R 2.4.0, the R Foundation for Statistical Computing). Antibody titers were analyzed with a general linear mixed model (Gaussian) with effects modeled as in cell culture time-course data ('lme' function, R 2.4.0). Percent anti-WNv_E-IgG and oral swab positive data were tested by Fisher's exact test. The fraction of days a bird was oral swab positive was tested by 2-way ANCOVA. These tests were performed with SAS JMP IN 5.1.2 (Cary, NC).

Results

Stress Response

Birds exposed to CORT excreted more FGM (Figure S1) and exhibited higher H:L ratios (Figure S2) than vehicle-controls ($P < 0.0001$ for both endpoints), but SR exposure did not impact these measurements.

Viral Shedding

Corticosterone treatment elevated viremia 8 – 1791 fold over controls (mean, 83; median, 46) on 2 DPI and extended it by 2 days (44% and 22% WN viremia positive on DPI 3 and 4, respectively) compared to controls (Figure 1a and Table). Fifty percent of the birds treated with SR alone were viremic one day longer than controls (3 DPI vs 2 DPI, respectively, $P = 0.0163$) (Figure 1a and Table). CORT+SR treated birds cleared viremia 1 day sooner than birds treated with CORT alone (0 vs. 22% on 4 DPI, respectively) but this was not statistically significant. CORT was the sole driver for differences in total viremia ($P = 0.0010$, Figure 1a). Mean viremia was highest among the CORT-treated birds on 1 DPI ($10^{4.10}$ PFU/ml serum (4.10 PFU)) compared to vehicle controls mean viremia of 3.22 PFU on the same day. CORT-treated birds experienced a peak viremia of 4.95 PFU on 2 DPI and vehicle-controls reached a peak 4.05 PFU on 1 DPI. Differences between these groups were more dramatic on 2 and 3 DPI, with mean viremia on 2 DPI of 4.05 vs. 2.14 PFU and on 3 DPI of 2.41 vs. 0.00 PFU in CORT vs. vehicle treated birds, respectively. No birds were viremic on or after 5 DPI.

Viremia-days (VD = mean PFU of a bird for all days that the bird was viremic * # of days that the bird was viremic) were then calculated, and compared to vehicle-treated birds (mean VD 4.80), CORT most significantly boosted VD (mean VD 9.08, $P < 0.0001$), SR did not (mean VD 6.08, $P = 0.5557$), and the interaction between these two treatments was not statistically significant (mean VD 8.38, $P = 0.0729$) (Table).

West Nile virus was detectable on oral swabs for up to 5 DPI. Cumulatively, vehicle, SR, CORT and CORT+SR treated-birds shed live WNv in 16, 20.4, 44.4 and 58.7% of oral swabs taken, respectively (Figure 1b). Corticosterone-treated birds shed virus orally for more days (DPI 1 to 5) than vehicle (DPI 2 to 5) or SR (DPI 1 to 3) treated birds; swabs were not collected on 6 DPI. When comparing the level of oral shedding between groups from DPI 2 – 4 (days of largest group-wise differences), we found that more CORT+SR-treated birds shed oral virus than CORT-treated birds and than all others ($P = 0.0079$ and $P < 0.0001$, respectively) but vehicle controls and SR treated birds did not statistically differ by this measure ($P = 0.4449$) (Figure 1b). We assessed overall (1-5 DPI) treatment effects on the fraction of days a bird was oral-swab-positive and adjusted this to the number of days a bird was living because 2 SR-treated birds were sacrificed for further study. We found that SR ($P = 0.0409$) and CORT ($P < 0.0001$) significantly augmented the fraction of days a bird was alive with oral virus, as did the interaction of SR and CORT ($P = 0.0239$) (Figure 1c).

Antibody to West Nile virus

WNv_EIgM (IgM) activity was first detected on 3 DPI (Figure 2a) whereas WNv_EIgG (IgG) was not detected until 4 DPI (Figure 2b). Both IgM (Figure 2c) and IgG

(Figure 2d) did not increase in quantity until 4 DPI. For both isotypes, all birds peaked at 10 DPI followed by a consistent drop in IgM by 14 DPI, but no change or a slight drop of IgG titers by 14 DPI.

Calculating the percent of birds with IgM and IgG reactive to WNv E protein in ELISA, we found that there were no treatment-related patterns in IgM production, but treatment greatly impacted IgG development. IgM activity was first detected on 3 DPI in 10% of the birds of each treatment group except CORT+SR treated birds; most birds were IgM-positive by 4 DPI. Corticosterone greatly accelerated IgG seroconversion compared to vehicle-controls (77.5% compared to 30% becoming IgG-positive on 4 DPI, respectively) (Fisher's Exact Test, CORT > vehicle, $P = 0.0142$). Thereafter, all CORT-treated birds and vehicle-controls were IgG-positive. SR treatment dramatically attenuated IgG production. On 4 DPI, only 10% were IgG positive to WNv_E compared to 30% of controls; on 5 DPI, 18% were IgG-positive compared to 100% of controls; 70% were positive on 7 DPI, and on 10 DPI, 100% seroconverted (Fisher's exact test, SR < vehicle, $P = 0.0005$). In summary, SR treatment delayed IgG seroconversion for 5 days compared to controls, but CORT treatment advanced it by one day.

During the days of greatest treatment-wise differences (IgM, 3-10 DPI; IgG, 4-14 DPI), IgG titers varied more strongly with treatment than IgM. CORT enhanced both IgM (Figure 2c) and IgG (Figure 2d) quantity ($P < 0.0001$) whereas SR suppressed IgG ($P < 0.0001$) but did not affect IgM levels ($P = 0.4522$). There was no interaction detected between CORT and SR in the production of IgM or IgG. At 14 DPI, IgM levels were the same between all treatment groups, whereas IgG levels in CORT-treated

birds' statistically matched vehicle controls, and SR-treated birds exhibited lower IgG levels compared to all others.

The finding that CORT treatment accelerated seroconversion prompted us to compare the present data to a pilot study (Jankowski, Unpublished data) in which sheep RBCs (SRBCs) rather than WNv were used as the test antigen. We found that antibody to SRBCs was significantly depressed on 6 DPI (Figure S3a) and that bursa weights responded to CORT treatment equally in both experiments (Figure S3b). We hypothesized that antibody (IgM or IgG) production was directly related to PFU and found a strong positive correlation; the amount of virus present correlated with IgM more strongly than with IgG (R^2 = 0.67 vs. 0.38, respectively) (Figure 3 shows IgM data).

Relationship between viremia and fecal corticosterone

Fecal glucocorticoid metabolites measured on the day of maximum viremia correlate the maximum viremia ($F=83.8$, $R^2=0.69$, $P < 0.0001$) (Figure 4).

Discussion

Our findings demonstrate that corticosterone elevation and pyrethroid exposure in chickens led to an altered capacity to immunologically respond to WNv infection. Viremia, percentage of birds that shed oral virus (Figure 1), and antibody response (Figure 2) varied with elevated corticosterone and waterborne exposure to SR. Compared to controls, birds exposed to corticosterone exhibited a higher viremia, produced higher antibody titers (IgM and IgG), and more birds shed oral virus, whereas SR-exposed birds had similar levels of viremia, but were viremic one day longer, and produced less antibody (IgG). We observed an interaction between CORT and SR exposure that led to more birds with oral WNv than birds treated with CORT or SR alone.

Immunotoxicity of Synergized Resmethrin

We aimed to expose the chickens to an environmentally relevant scenario in which adulticides are sprayed every other day for 3 days, leading to transient SR residues in water. With the SR concentrations used in this study (50 ppb resmethrin and 150 ppb PBO) and the estimated low SR dose (≅0.03 μg/kg or $6x10^{-9}$ X resmethrin's LC_{50}-bobwhite quail of >5000 mg/kg, USEPA 2006), we predicted interactive immunosuppressive effects with CORT, but minimal specific immunotoxic effects. Compared to controls, we found that SR extended viremia, enhanced oral shedding of WNv in CORT-treated birds, and suppressed WNV_EIgG production. However, with SR's small effect on viremia duration, despite its effect on greatly reducing IgG production, a study in which similarly treated chickens receive a second

WNv inoculation would help to better understand the importance of antibody suppression in altering the chicken's susceptibility to WNv.

In a variety of experimental models and concentrations, pyrethroids have been found to produce no effects on immunity (rats exposed to mg/kg permethrin for 28 days (Institoris et al 1999)), immunosuppression (lower cytokine production *in vitro* in mice exposed at 0.1% & 1.0% LD_{50}, (Diel et al 1998, 1998 and Blaylock et al 1995, respectively)) or immunostimulation (higher antibody forming cells in rats exposed to mg/kg deltamethrin (Madsen et al 1996)). These discrepancies might be due to concentration differences or species differences in gastro-intestinal absorption or metabolism of pyrethroids. We suggest that the combined effects of species differences in pyrethroid metabolism and chemical exposure regimes account for these contrasting results because pyrethroids are orally bioavailable to mammals (Miyamoto 1976) and birds (Christopher 1985). Considering that published studies have shown immunostimulation after mg/kg pyrethroid exposures and that the current study resulted in immunosuppression at μg/kg pyrethroid exposures, a hypothesis to explain this is that low levels (μg/kg) of pyrethroid are immunosuppressive and high levels (mg/kg) are immunostimulating. Such immunomodulation may occur through the alteration of cytokine profiles, as Diel et al (1999) reported pyrethroid-induced IL-4/INF-γ ratio shifts in lymphocyte culture depending upon exposure pathway. Perhaps different pyrethroid concentrations differently affect this ratio leading to either antibody suppression (reduced ratio) or antibody stimulation (increased ratio). The former would account for the current observations (Figure 2), while the later would provide a basis for the reported allergenic effects of pyrethroids (Hoellinger et al 1987, Diel et al 1998).

Corticosterone's impact on the immune Response to WNv

Our observation that H:L ratio, oral shedding, viremia and antibody were all higher than controls in CORT-exposed birds may be explained by understanding that, in general, CORT causes a shift away from immunity focused on intracellular pathogens to extracellular agents through a polarization from T_H1 to T_H2 cytokines (Daynes and Araneo 1989b). Specifically, IFN-γ (a key T_H1 and WNv cytokine (Shrestha et al 2006)) falls, whereas IL-4 (a major T_H2 cytokine) rises upon CORT exposure. CORT also causes heterophils (avian phagocytic cells) to exit and lymphocytes to enter subcutaneous tissues, leading to an increased H:L ratio in the blood (Figure S2). It is plausible that the higher viremia and thus antigenic stimulus caused by CORT's presumed immunosuppressive effects on innate immunity, led to enhanced IgM, and this, coupled with a shift towards T_H2 type responses explain the enhanced level of IgG. Perhaps the signal provided by a replicating antigen to the immune system obscures the negative impact of CORT on antibody production because CORT significantly depressed antibody levels in chickens exposed to non-replicating SRBC antigens (Figure S3). The finding that CORT-treated birds exhibited higher viremia and antibody levels may indicate the relative unimportance of antibody compared to innate immunity upon primary WNv infection in birds or that the antibody generated or measured was not specifically neutralizing to WNv.

Impact of Corticosterone and SR on Oral Shedding

Corticosterone increased oral shedding of WNv (69% of CORT-treated birds were oral swab positive vs. 26% of control birds on 2-4 DPI). Comparing CORT- to

CORT+SR-treated birds, there was a consistent increase (53% vs. 86% positive for DPI 2 – 4, respectively) in oral shedding by CORT+SR-exposed birds. This might be due to a physically destructive effect of SR on infected cells lining the oral mucosa leading to leakage of intracellular contents into the oral cavity. Perhaps in conjunction with this effect and given SR's capacity to reduce IgG production, SR may have reduced the production of IgA and reduced sequestration of WNv in the mouths of CORT+SR treated birds.

An Environmental Perspective

Whether the viremia levels determined in this study would lead to more infected mosquitoes is an important question. Some studies might suggest viremia levels below 5 PFU would not infect an epidemiologically important number of mosquitoes (Chamberlain et al 1954, Komar et al 2003). However, given the hypothesis that the probability of infecting a mosquito continuously and positively relates to viremia, even small enhancements in viremia magnitude (CORT exposure) or duration (CORT or SR exposure) could lead to proportionally more infected mosquitoes, depending upon which portion of the infection-probability curve a viremia level falls (Lord et al 2006). Thus, viremia levels in the range found in this study (up to 4.95 PFU) may lead to an important increase in mosquito infections. Caution is encouraged when placing these viremia levels into an epidemiological context, however, because although 4.95 PFU may be somewhat infectious to mosquitoes, this significantly depends on the mosquito vector and the virus strain (Reisen et al 2008). It may be that despite elevated CORT levels, the chicken will not become highly infectious, but that more susceptible avian hosts

experiencing similar CORT elevations would transition from being moderately to highly infectious to mosquitoes. Environmental stressors may cause an incidental host to become an amplification host.

Although the FGM levels in the CORT-exposed birds in this study seem extraordinarily high (8-12 times controls), according to previous work in which chickens were treated as in the current study, plasma corticosterone levels would have been approximately 25 ng/ml on the day of WNv inoculation (Post et al 2003). In that study, this was ~5 times basal and similar to a low level ACTH (1 IU/kg) stimulation. This is likely not beyond what could be observed in birds experiencing medium-term environmental stressors. Future studies of variably-housed chickens or of wild birds residing in varying levels of habitat quality may consider the assessment of species specific FGM reference ranges in order to better determine how different FGM levels relate to viremias found in laboratory WNv-infection trials.

Lastly, with pyrethroid resistant mosquitoes continuing to emerge (Brogdon and McAllister 1998, N'Guessan et al 2007), varied efficacy (Reddy et al 2006, Carrey et al 2008) and the currently-demonstrated immunosuppressive impacts on avian hosts, further work should aim to place these findings into an epidemiological context. For example, the toxicant levels used in this study were 171 (resmethrin) and 2.5 (PBO) times more than what has been found in water after adulticiding campaigns for WNv (Abbene et al 2005; also see Schleier et al 2008) but 10% of what has been found in sediments near agriculture (Weston et al 2004). The relationship between pyrethroid concentrations before, during and after adulticiding campaigns in environmental media

that are potentially consumed by different species of birds compared to their immune response against WNv and other arboviruses should be investigated.

Broadly speaking, "stressed" birds may constitute a wildlife and public health threat through a reduced capacity to immunologically contain WNv replication, leading to a more transmissible viremia and thus a more infectious local environment. Perhaps stress-reducing land-use practices would serve as an environmental vaccination such that healthier birds better contain WNv transmission and thus mitigate WNv hotspot emergence. Future investigations of the relationships between environmental stressors and intraspecies variation in avian host immunocompetence may yield novel insights into WNv epidemiology.

Conclusions

This study demonstrated that oral exposure to 10 days of 20 mg/l CORT or three alternate days of 50 ppb resmethrin/150 ppb PBO via drinking water suppressed immunity to WNv in chickens, and that subacute exposure to elevated corticosterone enhanced the immunotoxicity of SR to one measure (oral virus shedding). CORT exposure more significantly increased viremia whereas SR exposure reduced IgG production. Further work to place these findings into an environmental and epidemiological perspective is warranted.

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Table. Detailed West Nile viremia profiles (PFU/ml[%positive]) and consequent mean ± SEM viremia-days (VD = mean PFU/ml x days viremic).

	Treatment Group ('effect')			
	Vehicle (*n*=10)	SR (n=11, 10)	CORT (*n*=9)	CORT+SR (*n*=10)
1 DPI	1.70-4.05[100]	2.30-3.56 [92]	2.60-4.83[89]	3.78-4.86[100]
2 DPI	1.70-2.70[80]	1.70-2.90 [83]	3.30-4.95[100]	3.20-4.32[100]
3 DPI	0[0]	1.85-2.60[50]	1.70-3.18[44]	1.70-2.18[40]
4 DPI	0[0]	0[0]	1.70[22]	0[0]
VD[||]	4.80±0.50	6.08±0.52	**9.08±0.52	*8.38±0.55

|| Whole model results: $F = 15.5252$, $R^2 = 0.64$, $P < 0.0001$.

**$P < 0.0001$

*$P = 0.0729$

Figure Legends

Figure 1.

Viral shedding profiles as determined by Vero cell culture. (a) Mean ± SEM viremia. Viremia statistics summarize total viremia response curves. ^ Indicates that SR-treated birds were viremic one day longer than vehicle-controls ($P = 0.0163$). * Indicates that $P = 0.0010$ for the main effect, CORT. (b) Percent of birds within a treatment group that were shedding oral virus on a given DPI. Fisher's Exact Test was used to compare treatment groups. (c) Mean ± SEM fraction of days a bird shed virus while it was alive. 2X2 ANCOVA was used to compare means. $P < 0.05$ between different letter superscripts.

Figure 2.

WNV_EIgM and WNV_EIgG profiles. (a) IgM and (b) IgG were considered WNV_E-positive by ELISA when the OD of sample wells were > 2.0 times the OD of negative control wells. The $\log_2$ titers of (c) IgM and (d) IgG were calculated by inserting a sample's OD into a dilution curve of WNV_E-antibody positive chicken sera. $P < 0.0001$ between different letter superscripts.

Figure 3.

Correlation between WNV_EIgM and maximum level of viremia ($F=69.6$, $R^2=0.67$, $P < 0.0001$). The correlation between WNV_EIgG and maximum level of viremia is not shown ($F=21.3$, $R^2=0.38$, $P < 0.0001$).

Figure 4.

Correlation between fecal glucocorticoid metabolites and maximum West Nile viremia ($F=83.8$, $R^2=0.69$, $P < 0.0001$).

Figure 1.

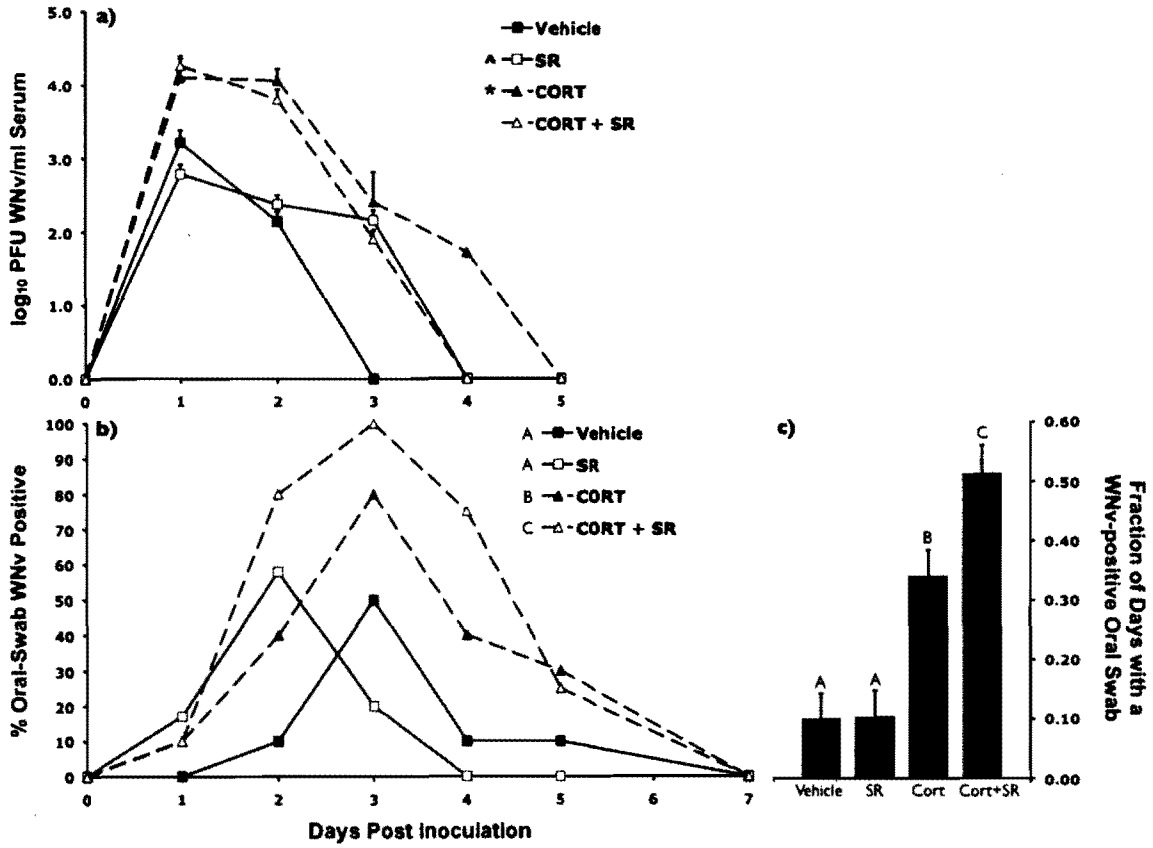

a)
Vehicle
SR
CORT
CORT + SR
log10 PFU WNv/ml Serum
b)
A Vehicle
A SR
B CORT
C CORT + SR
% Oral-Swab WNv Positive
Days Post Inoculation
c)
Vehicle
SR
Cort
Cort+SR
Fraction of Days with a WNv-positive Oral Swab

Figure 2.

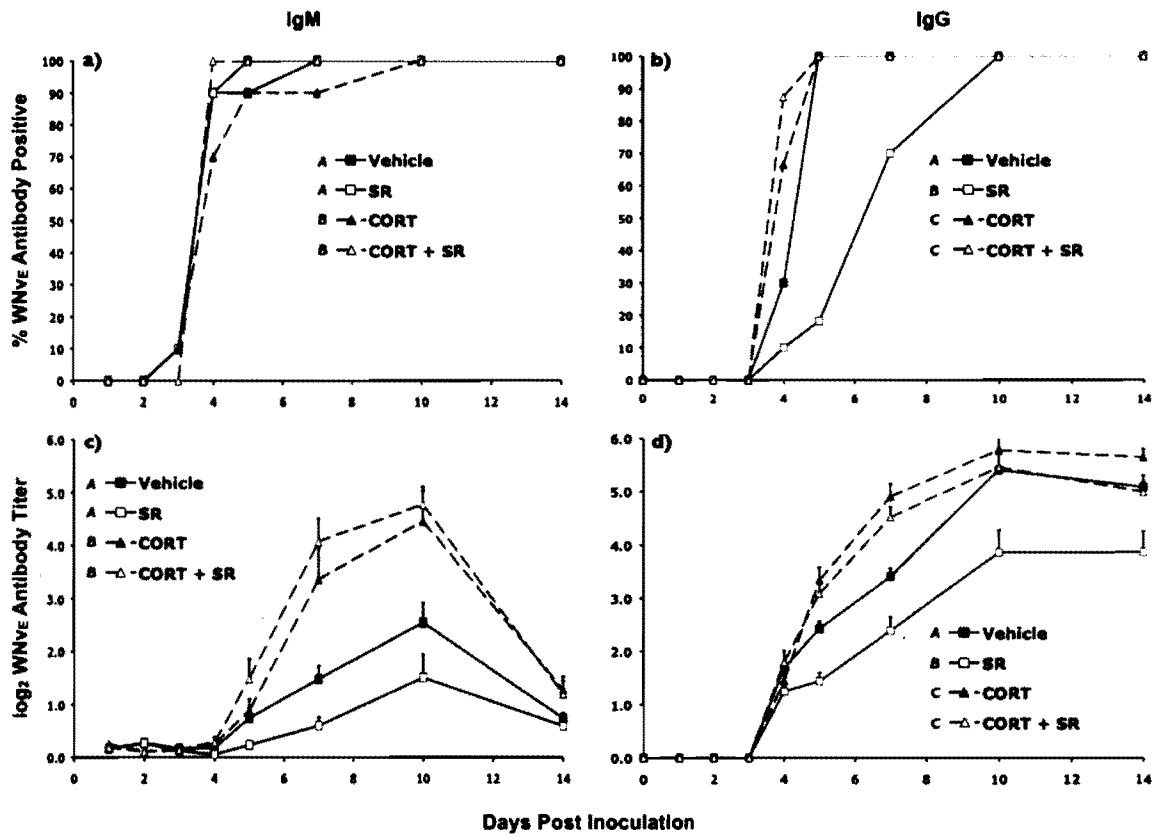

IgM
IgG
a)
b)
c)
d)
% WNvE Antibody Positive
log2 WNvE Antibody Titer
Days Post Inoculation
A Vehicle
A SR
B CORT
B CORT + SR
A Vehicle
B SR
C CORT
C CORT + SR

Figure 3.

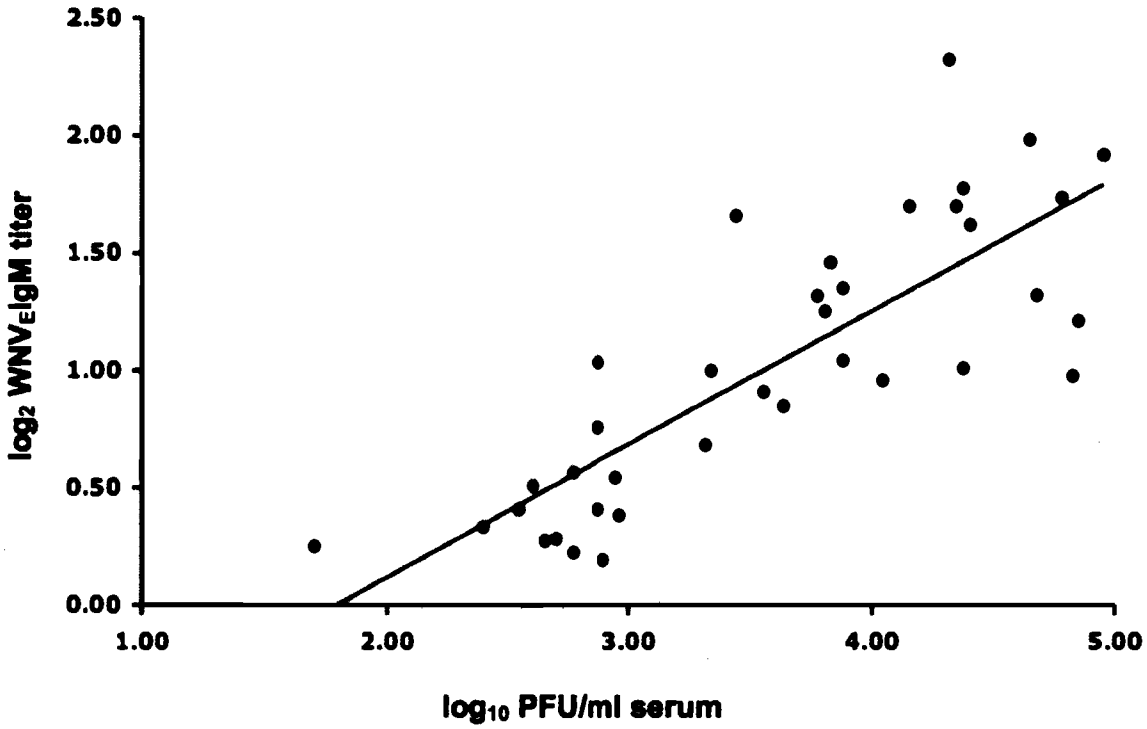
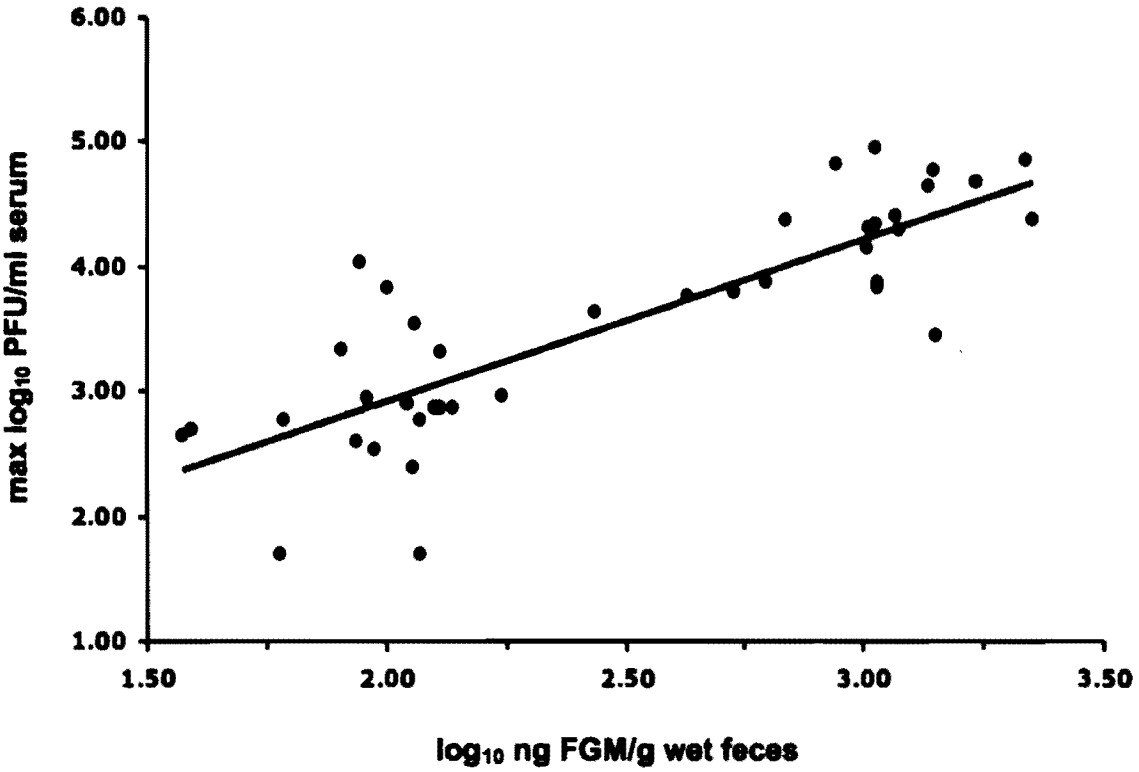

2.50
2.00
1.50
1.00
0.50
0.00
log2 WNVEIgM titer
1.00
2.00
3.00
4.00
5.00
log10 PFU/ml serum

Figure 4.


6.00
5.00
4.00
3.00
2.00
1.00
max log10 PFU/ml serum
1.50
2.00
2.50
3.00
3.50
log10 ng FGM/g wet feces

Supplemental Material

Supplementary Figure Legends

Figure S1.

Corticosterone administration but no other treatments affected the (a) magnitude (mean experimental phase fecal glucocorticoid metabolite (FGM) / baseline FGM) and (b) concentration of chicken FGM. Data are shown according to experimental phase (1 = baseline; 2 = CORT administered; 3 = CORT + SR administered; 4 = CORT + SR + WNV administered; 5 = SR removed; 6 = all treatments removed) plotted by treatment group. Linear mixed effects model results: SR, $P = 0.3322$; CORT, $P < 0.0001$; CORT*SR, $P = 0.2332$.

Figure S2.

Corticosterone administration but no other treatments affected the heterophil:lymphocyte (H:L) ratio (by blood smear). Poisson-linked generalized linear mixed model results by effect: SR, $P = 0.6666$; CORT, $P < 0.0001$; CORT*SR, $P = 0.7916$.

Figure S3.

Mean ± SEM antibody (hemagglutination assay) at 6 DPI to sheep red blood cells (SRBCs) was reduced by treating with 10 days of corticosterone (20 mg/l) in drinking water ($P = 0.0005$).

Figure S1.

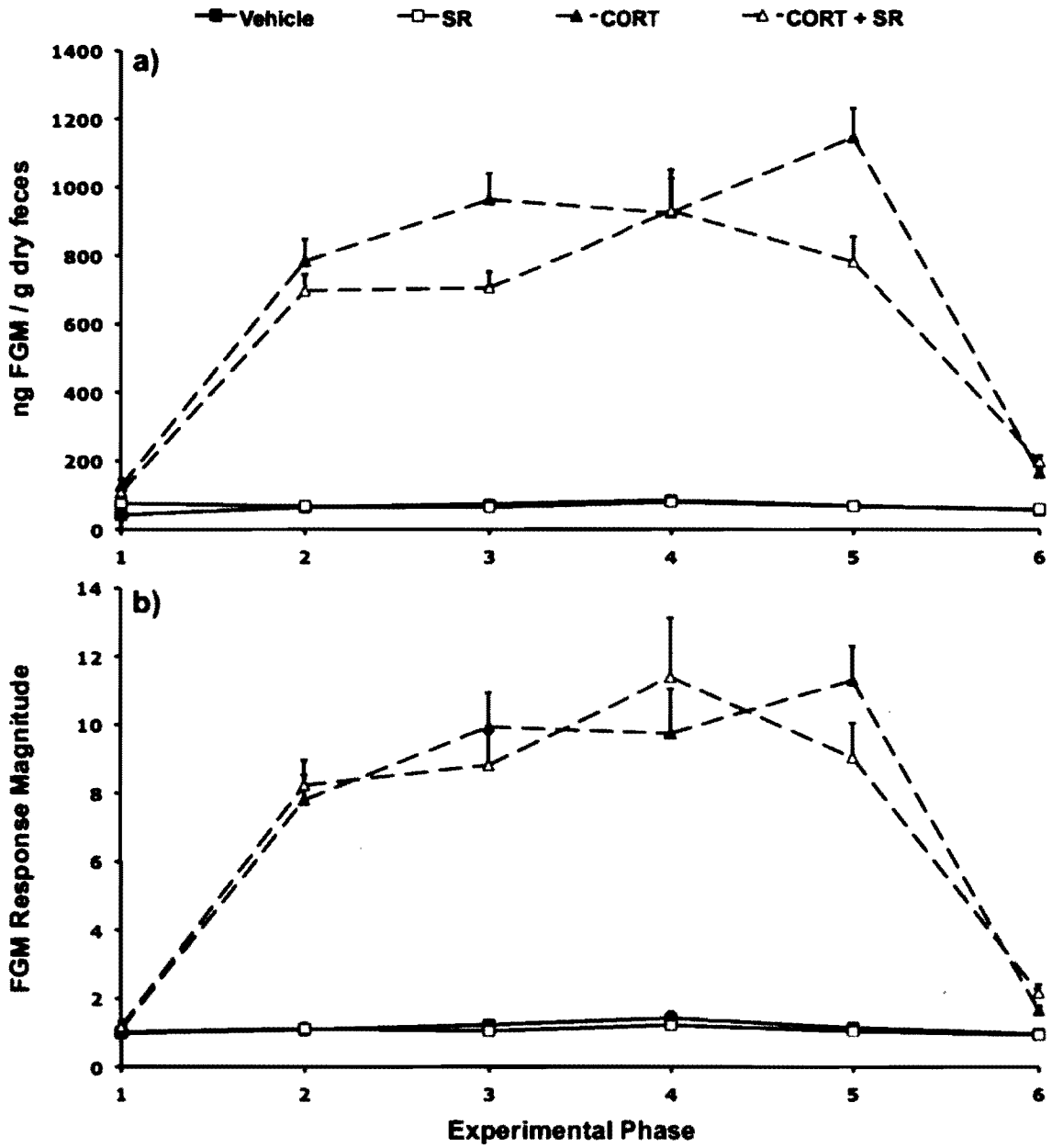

Vehicle
SR
CORT
CORT + SR
a)
ng FGM / g dry feces
1400
1200
1000
800
600
400
200
0
1
2
3
4
5
6
b)
FGM Response Magnitude
14
12
10
8
6
4
2
0
Experimental Phase

Figure S2.

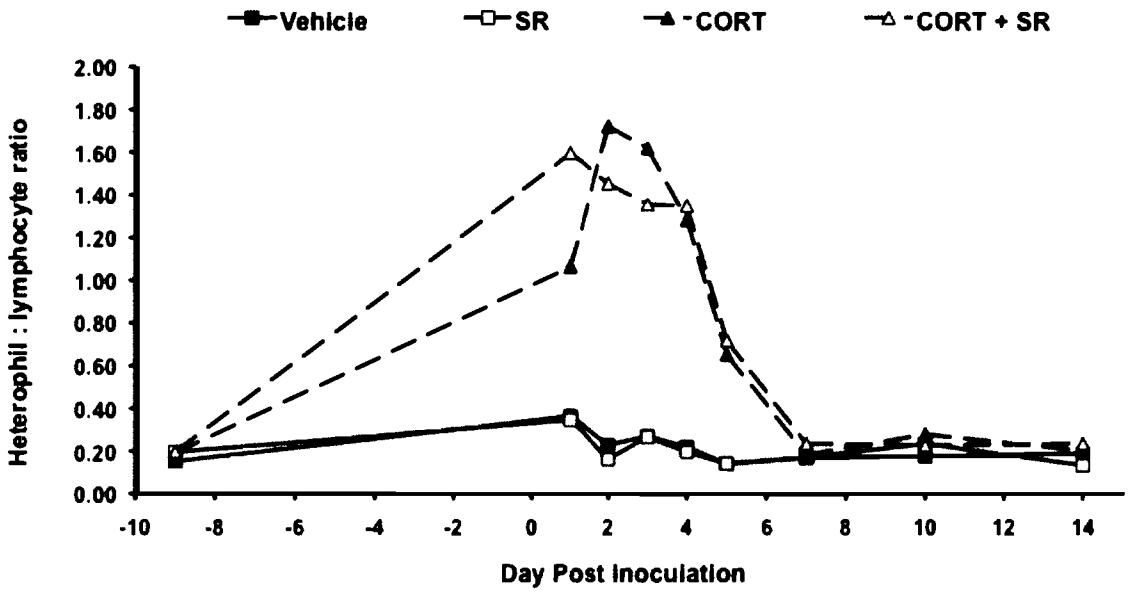
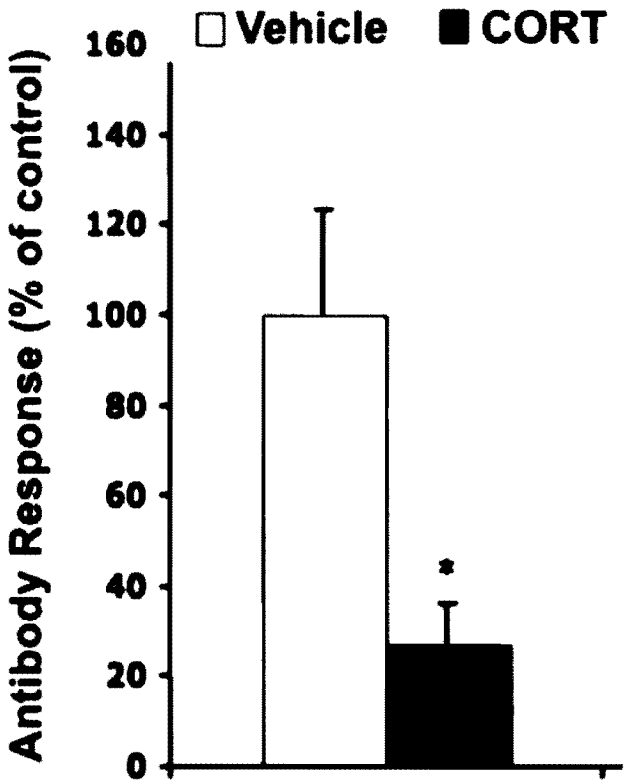

Vehicle
SR
CORT
CORT + SR
Heterophil : lymphocyte ratio
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
-10
-8
-6
-4
-2
0
2
4
6
8
10
12
14
Day Post Inoculation

Figure S3.


Vehicle
CORT
Antibody Response (% of control)
160
140
120
100
80
60
40
20
0
*

Supplementary Material – Methods

Anti-WNv Envelope Protein IgG Assay protocol

For this assay, 96-well microtiter plates (Nunc Maxisorp, #430341, Rochester, NY) were coated with an anti-flavivirus monoclonal antibody (MAB4G2, CDC, #0035) diluted 1:5000 in coating buffer (pH 9.6) and incubated overnight at 4°C. The plates were tapped to remove antibody and then blocked (Carnation Instant Nonfat Dry Milk®, Vevey, Switzerland) for 30 minutes at room temperature. Wells were then washed 4X with wash buffer (pH 7.2). Recombinant COS-1 cell culture antigen (WNv_E, Hennessy Research, #P120-1, Lot. 10XP040103, Shawnee, KS) was diluted 1:100 in wash buffer, added to all wells and allowed to react overnight at 4°C. Plates were washed 4X with wash buffer. Chicken serum samples were pooled according to DPI and compared to one another to select high and low controls. High (14 DPI) and low (4 DPI) positive and negative (-9 DPI) serum pools were added in quadruplicate while serum samples were added in duplicate at 1:200 in wash buffer and incubated for 1-hour at 37°C, 5% CO_2. To estimate a standard curve, high positive controls were serially diluted two-fold from 1:100 to 1:25,600. After incubation, wells were washed 4X with wash buffer, then treated with goat anti-chicken-IgG-HRP (KPL, #14-24-06) diluted to 1:1000 in blocking buffer for 1 hour at 37°C. Plates were then washed 4X with wash buffer. The enzyme substrate ABTS (KPL, #50-62-00) was added, and the plates were incubated for 10 minutes in the dark. The reaction was stopped with 1% SDS and optical density was measured at 405 nm. Serum samples were considered WNv_E-antibody positive if the ratio between its average optical density (OD), and the average OD of the negative

control was greater than 2.0. IgG titers for positive samples were then calculated from the standard curve's optical density versus dilution slope. Intra-assay coefficients of variation were 8.74% and 6.24%, while inter-assay coefficients of variation were 15.94% and 4.73% for high and low pools, respectively.

Anti-WNv Envelope Protein IgG Assay protocol

Changes to Johnson et al (2002) included: coating antibody (goat anti-chicken IgM, #643951, MP Biomedicals) was diluted to 1:400 in wash buffer; serum was added at 1:200; recombinant COS-1 produced envelope (E) protein (Hennessy Research) was used at 1:100 in wash buffer as the antigen. Monoclonal antibody 6B6C-1 (MAB8744, Millipore, Billerica, MA) was used at 1:2000 in blocking buffer. The reaction was detected by reacting with TMB (KPL, #50-76-00) for 25 minutes and stopped with 1 M H_3PO_4 and OD was recorded with a microplate reader at 450 nm.