Supplementary Materials for

Title: Light Pollution Increases West Nile Virus Competence in a Ubiquitous Passerine Reservoir Species

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Materials and Methods

Experimental Procedures

House sparrows were captured using mist nets at two sites in the Tampa Bay area with comparable levels of light pollution as determined by satellite imaging and handheld light meters (1). All birds were captured between the hours of 5:30 and 9:30 AM. Males and females were evenly distributed throughout treatments to account for difference among sexes. Additionally, all birds captured were adults (i.e. between 1-3 years of age). To assess how light pollution affects the hypothalamic-pituitary-adrenal HPA axis, we performed dexamethasone (DEX) suppression tests using methods described below on each bird immediately after capture in the field and after ALAN/control lighting exposure. Birds were then transported to the University of South Florida vivarium where they were housed individually in 13"x15"x18" cages for the next 7-25 days in visual and audial proximity to each other. Control birds were exposed to ~0 lux at night and kept on 12h light:12h dark cycle consistent with late spring in Florida for the project duration. All ALAN birds were exposed to ~8 lux of incandescent white light during what was the dark period for control birds (12h light:12h dim light). Food (mixed seeds) and water were provided *ad libitum* throughout the study and IACUC (#2716) and USF Biosafety (#1323) approved the studies prior to the work.

Two days prior to WNV exposure, all birds were administered second DEX suppression tests according to the below bleeding timeline and procedures. The next day, all birds were transported to the USF Biosafety level-3 (BSL-3) suite where they were kept singly in similar cages but inside bioBUBBLE containment systems (bioBUBBLE Inc, Fort Collins CO) to prevent WNV escape into rooms. Light conditions during this period were identical to conditions described above. One day after acclimation to the BSL-3 facility, all birds were inoculated with 10^1 plaque-forming units (PFUs) of New York 1999 strain at ~5 minute intervals between 7 and 9 am to account for any effect of inoculation time on competence (NY 1999; Gervasi, Burgan, Hofmeister, Unnasch, & Martin, 2017). Due to space constraints in the BSL-3 facility, this study was conducted in two cohorts, but all birds from both cohorts were inoculated using a common WNV stock.

Although we did not assess prior exposure to or current infection with WNV in these birds, unpublished research by our lab found that individuals are unable to be infected with WNV twice. We used infection as a proxy for whether individuals had any prior exposure to WNV. As all individuals became infected once they were exposed, we concluded that none of the individuals had any prior exposure to WNV.

Birds were sampled on days 2, 4, 6, and 10 at the same time of day following WNV inoculation. ~70 uL of blood was extracted using procedures described below. Bird mass was also measured prior to WNV inoculation, and during sampling periods on days 2, 4, 6, and 10 using methods described below. Mortality was monitored twice daily during infection period, and birds were

euthanized when expressing sickness behaviors, which are typically only expressed when death appears eminent in the near future. All birds were euthanized on d10 following inoculation using deep isoflurane anesthesia and rapid decapitation.

Sample Collection

Blood samples for the DEX suppression test required a baseline CORT sample, which was obtained within 3 minutes of capture, a post-stressor blood sample which was collected after 30 minutes of restraint in a cloth bag following initial capture, which was immediately followed by a DEX injection (s.q., 28ug dissolved in 50 uL peanut oil), and final samples were collected 1h after injections. Blood samples were collected from the brachial vein using sterile 26-gauge needles and microcapillary tubes, and serum was frozen at -20C until hormone assay.

Blood samples for viremia were collected using sterile 26-gauge needles and microcapillary tubes rinsed with sodium citrate to prevent clotting of blood. Serum was extracted from the blood samples and frozen at -20C until viral RNA extraction and qPCR.

Body mass

Body mass measurements were recorded using a Pesola spring scale. Mass was recorded to the 0.01 gram on the day of inoculation, and days 2, 4, 6, and 10 following WNV exposure.

RNA extraction and qPCR for WNV titer.

WNV RNA was extracted from 10uL of stored serum using the Qiagen QIAmp Viral Extraction Mini Kit (Qiagen Cat. No. 52906). Viremia was quantified using quantitative real-time polymerase chain reaction (qRT-PCR) using a one-step Taqman kit (iTaq Universal Probes One-Step Kit; Bio-Rad Cat. No. 1725141). Standards were extracted from known concentrations (via plaque-assay) of WNV stock and quantified using the same methods listed above. Forward and reverse primers and probe sequences are listed below (2). All samples were run in duplicate with negative controls.

Forward Primer: 5’ CAGACCACGCTACGGCG 3’
Reverse Primer: 5’ CTAGGGCCGCGTGGG 3’
Probe: 5’ [6~FAM] CTGCGGAGAGTGCAGTCTGCGAT [BHQ1a~6FAM]

Corticosterone Assays

Corticosterone concentrations were quantified in serum using an enzyme immunoassay (EIA) kit from Arbor Assays (Arbor Assays, Ann Arbor, MI, product # K014-H5; Gervasi et al., 2017). Samples were run in duplicate and standardized across plates. Concentrations were derived from known values along the standard curve, and all values fell within the curve.

Supplementary Text

Days in captivity results

Our study was designed to capture the effects of duration of exposure to ALAN on corticosterone and viremia by housing birds under their designated conditions for a range of 7-25 days. We added "days in captivity" as a fixed effect in the mixed model analysis using the nlme package in R studio and found that days in captivity had no significant effect on the models (P=0.8024). A second set of mixed models intended to determine the effect of days in captivity on CORT area under the curve (AUC) was unable to be run using the 'nlme' package or the 'lme4' package in R studio. After a series of diagnostic tests, we discovered the reason the mixed model was unable to be run was that the random effects explained almost all of the variance (i.e. there was no difference in the CORT AUC between treatment groups).

Corticosterone results

First, we queried effects of ALAN on baseline (i.e, prior to a stressor), post-restraint (i.e., after a 30-minute psychological stressor), and post-dexamethasone (i.e., a synthetic glucocorticoid that induces down-regulation of endogenous corticosterone release; Liebl, Shimizu, & Martin, 2013) concentrations, but there was little evidence that ALAN affected HPA function when all aspects were analyzed in a single model (treatment: $F_{1,40} = 2.8$, $P = 0.14$). HPA function changed over the course of the study (time: $F_{5,197} = 38.2$, $P < 0.001$; time x treatment: $F_{5,197} = 2.4$, $P = 0.04$), but most of this variation was due to captivity, which we have observed previously to affect HPA function in house sparrows (**Figs. S1 & S2**; Martin, Kidd, Liebl, & Coon, 2011). The only statistically significant effect of ALAN on HPA function was on baseline CORT (time x treatment: $F_{1,75} = 4.6$, $P = 0.03$); baseline CORT was lower just prior to WNV exposure in ALAN compared to control birds (**Fig. S3**).

Cell type enrichment results

Following a principal components analysis visualized in **Fig S4**, significant cell type enrichments are presented in **Fig S5.** Down regulated genes for both contrasts were strongly enriched for CD71+ Early Erythroid cells, an early precursor of red blood cells (RBCs). This down regulation occurs in ALAN birds at d6 relative to d2 and at d6 relative to Control. Additionally, up regulated genes in both comparisons are enriched for a wide variety of cell types, including many immune functioning cells (**Fig S6**). Thus, this represents a decrease in RBCs and increase in circulating lymphocytes. The down regulation of hemoglobin (Supplemental DEseq2 results) and up regulation of immune related genes in ALAN birds could result from a shift in cell type abundance. Nearly 3000 genes across several networks were differentially expressed and likely impacted the outcome of WNV infection in ALAN exposed individuals.

Body mass analysis

We have analyzed body mass throughout the course of infection using two models. The first model was a linear mixed model conducted in the nlme r software package where equal variances were assumed between groups. The dependent variable was body mass, the fixed effects were treatment, day, and their interaction, and the random effect was bird ID. There was a significant effect of treatment (P=0.0023), day (day4 P=0.0277; day6 P=0.0104) and their interaction (treatment*day6 P=0.0215) on body mass. A second mixed model using the same terms but allowed for variances to differ between groups was built; again, treatment (P=0.0021),

day (day4 P=0.0253, day6 P=0.0147) and their interaction (treatment*day6 P=0.0461) had a significant effect on body mass. We performed an ANOVA to compare the two models, but there was no significant effect of allowing for variance to differ on the linear mixed model (P=0.1392). We therefore chose to report the statistics from the linear mixed model that allowed for differing variances as a conservative estimate of the observed effects. See tables for details of models.

Collinearity Diagnostics

We performed collinearity diagnostics in R studio using the 'olsrr' package between treatment, day, and viremia (5). We used variance inflation factors (VIF) to detect any variance that may have been inflated by a collinear relationship between variables. VIF values above 4 demand further diagnostics, where values above 10 are strong signals of collinearity; there were two values that were between 4 and 10, so we conducted a follow-up Eigenvalue condition index diagnostic test. No two values had large variances denoted by Eigenvalue condition indices greater than 30, so we further concluded that there was no collinearity between variables in this model. See tables for detailed output information.

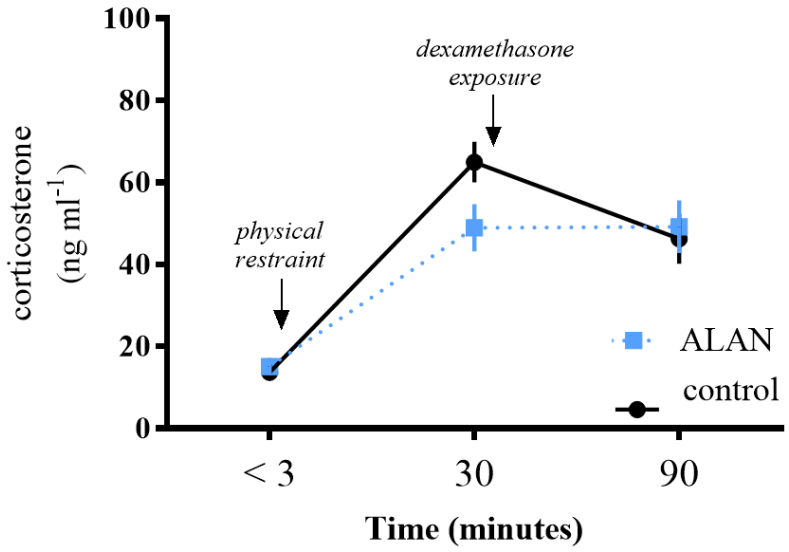

Fig. S1.

Corticosterone levels at capture in the wild: baseline (<3 min of hitting a mist net), 30 (after 30 min restraint in a cloth bag), and 90 (after DEX-induced negative feedback in ALAN (blue) and control (black) individuals. This regulatory profile represents the ability of birds to mount a corticosterone response to a stressor and respond to agonism of glucocorticoid receptors in the brain with attenuation of corticosterone release from the adrenals. Note that all of these values were collected before any individuals were exposed to ALAN.

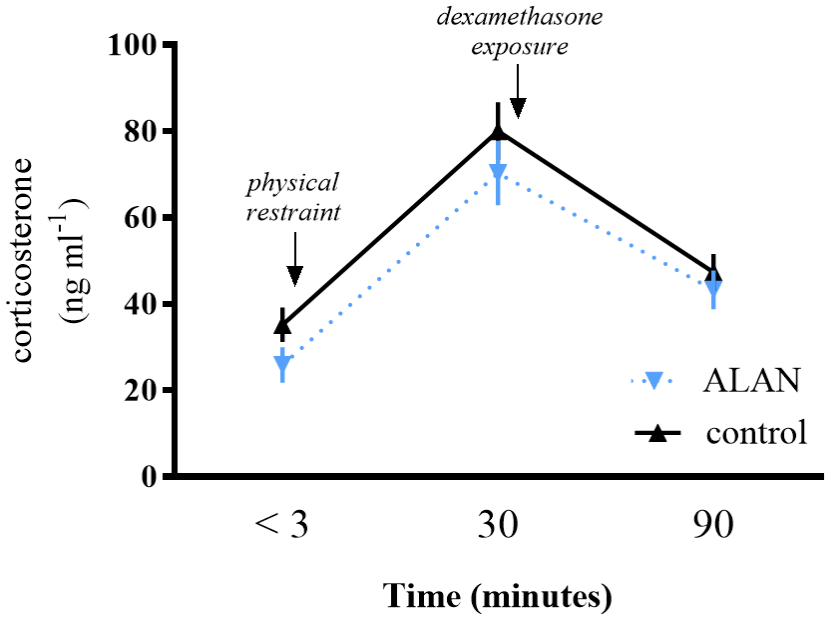

Fig. S2.

Corticosterone regulatory profiles after ALAN exposure; ALAN-exposed individuals are depicted as blue symbols and control individuals are black. As above in Fig. S1, < 3 depicts baseline measures, 30 minutes depicts post-stressor measures, and 90 minutes depicts post-DEX negative feedback measures.

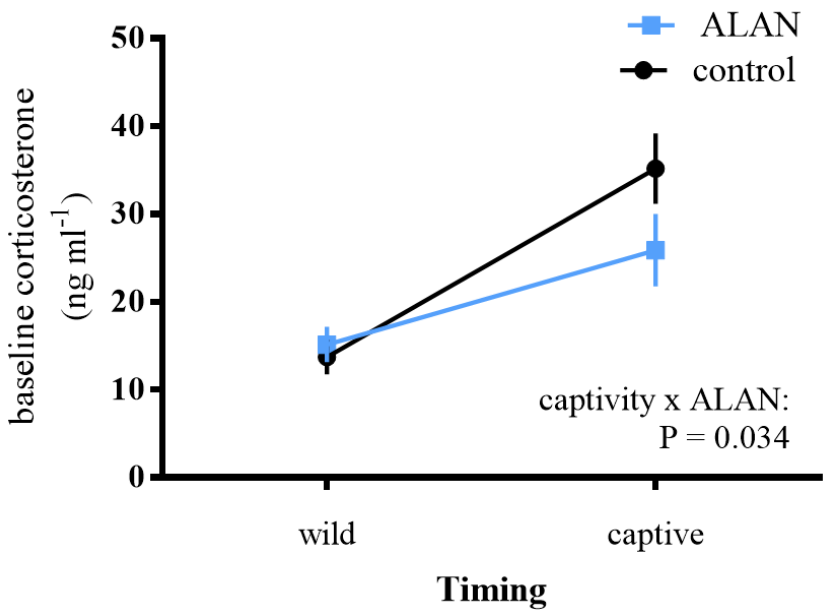

Fig. S3.

Baseline corticosterone levels of birds at capture (wild) and after a period of time in captivity (captive). As above, ALAN exposed birds are depicted in blue and control birds in black. Both groups increased baseline corticosterone after time in captivity, a typical response for this species (4), but this increase in baseline corticosterone was more modest in ALAN-exposed birds.

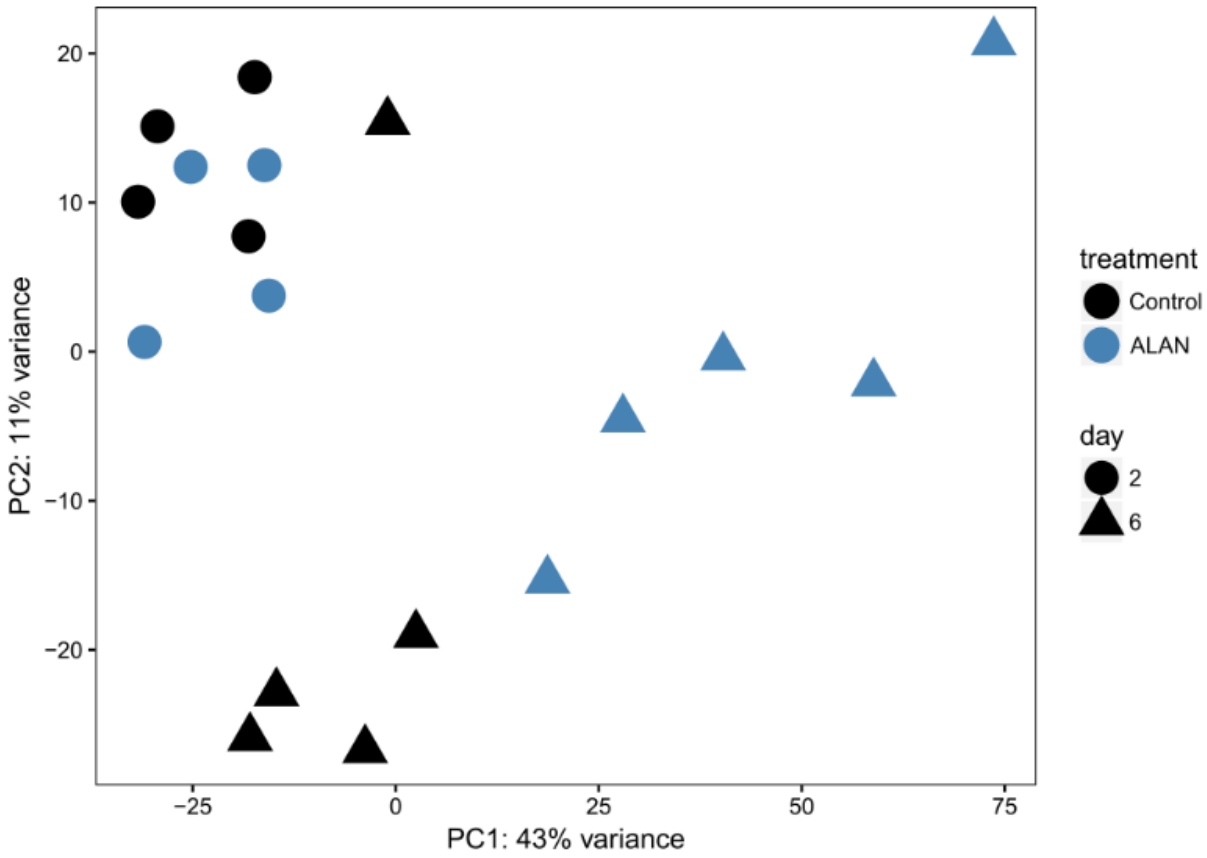

Fig. S4.
PCA of all 18 RNAseq libraries used in the study.

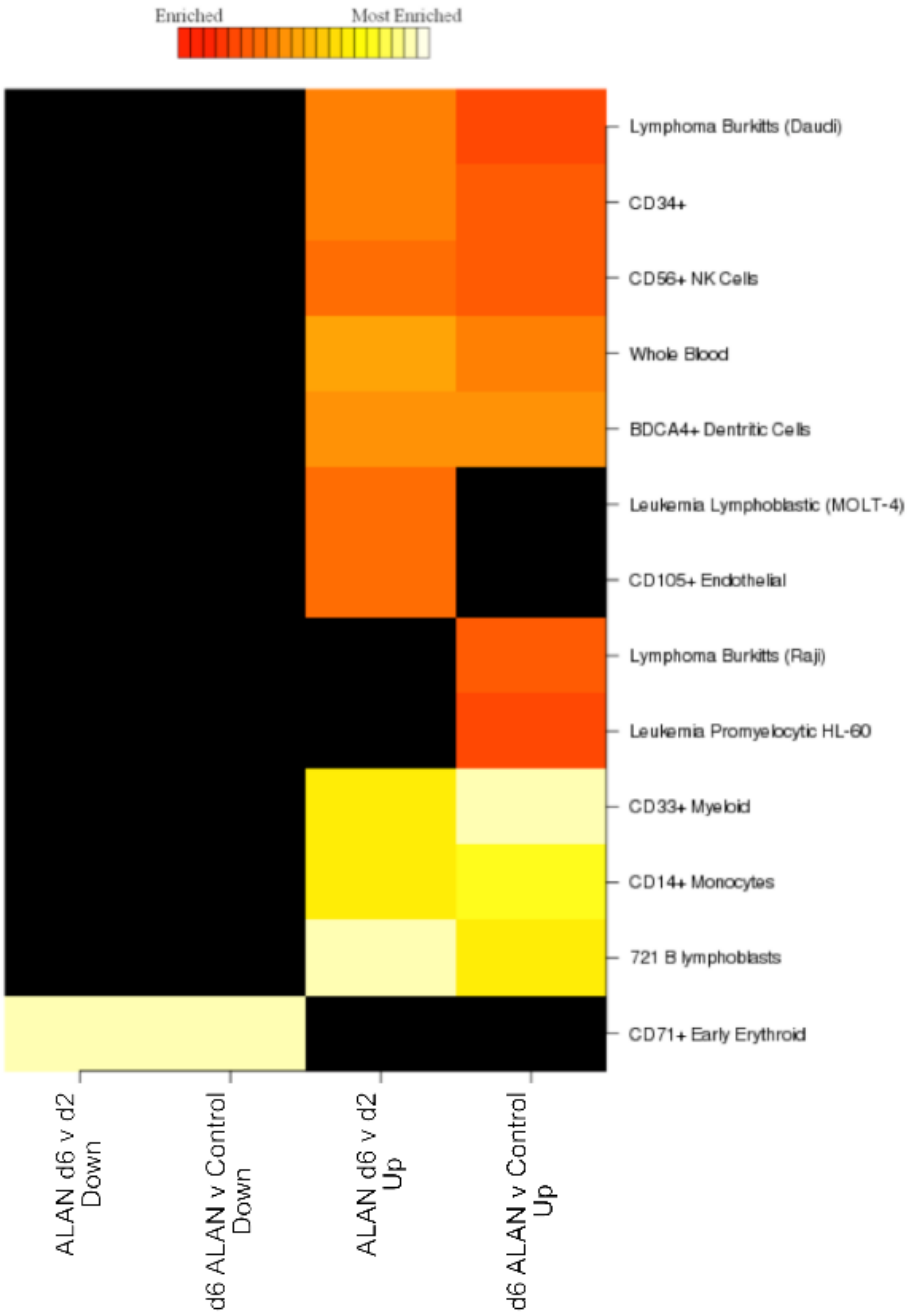

Fig. S5.
Cell type enrichment analysis based on up and down regulated genes in the ‘d6 ALAN v Control’ and ‘ALAN d6 v d2’ DEseq2 results. Only significant enrichments are shown, with lighter colors indicating a stronger enrichment.

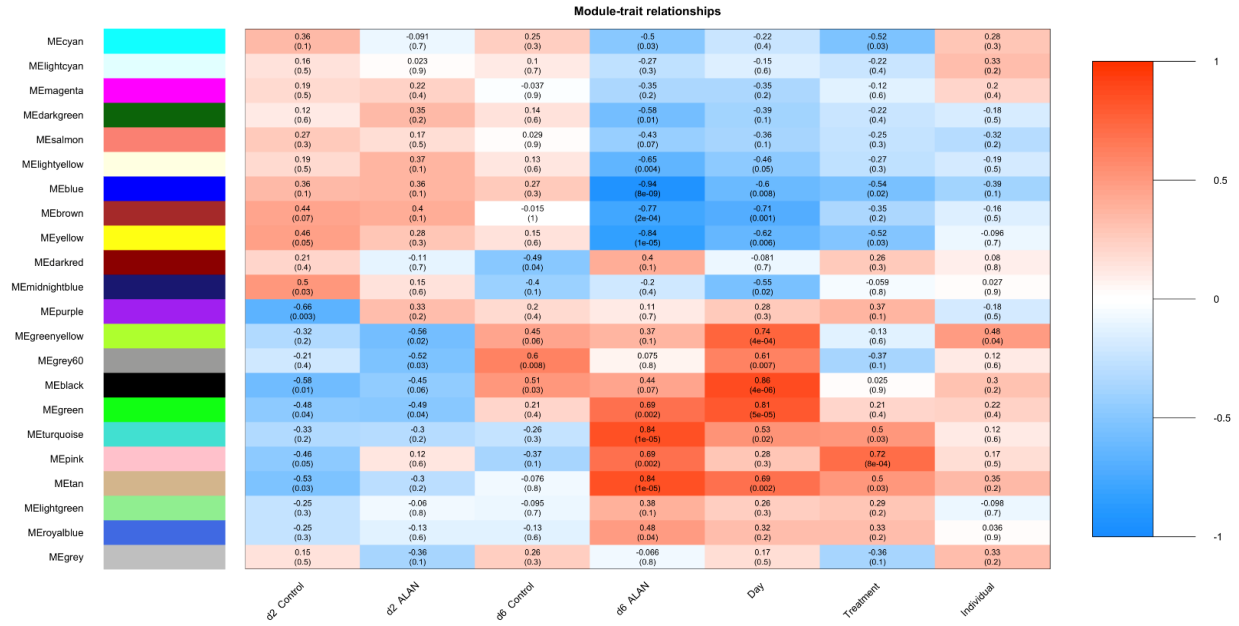

Fig. S6.
WGCNA module trait correlations. Each box contains the correlation value, ranging from -1 to 1, and corresponding pvalue. The heatmap color shading corresponds to the correlation value, with red colors representing positive correlations and blue colors representing negative correlations.

Table S1.

Terms used in the linear mixed model to determine effects of days in captivity on viremia. These two models were conducted with the 'nlme' package in R studio; the first model included days in captivity as a fixed effect and the second model removed this term. The ANOVA comparison revealed that these models did not significantly differ and that days in captivity (i.e. duration of ALAN exposure) did not influence viremia.

R package	Dependent Variable	Fixed Effects	Random Effects	AIC	BIC	LogLikelihood	P value
nlme	viremia	dayscaptivity+treatment*day	id	568.105	637.3499	-262.0525	0.8024
nlme	viremia	treatment*day	id	555.459	596.3767	-264.7296	

Table S2.

Parameters used for the survival analysis. This table shows the number of birds alive at each day throughout the course of infection; notice that mortality only occurs between days 4 and 8 post exposure.

Day	Treatment	Number Alive
0	Control	22
0	ALAN	23
1	Control	22
1	ALAN	23
2	Control	22
2	ALAN	23
3	Control	22
3	ALAN	23
4	Control	22
4	ALAN	23
5	Control	18
5	ALAN	19
6	Control	14
6	ALAN	14
7	Control	13
7	ALAN	10
8	Control	13
8	ALAN	10
9	Control	13
9	ALAN	10
10	Control	13
10	ALAN	10

Table S3.

Terms used in the linear mixed models to determine effects of ALAN, day post-exposure, and their interactions on body mass throughout the course of infection. These two models were conducted with the 'nlme' package in R studio; the first model assumed equal variance among groups and the second model allowed for unequal variance. The ANOVA comparison determined these models did not significantly differ, therefore, the statistics for the more robust model allowing for unequal variance were reported.

R package	Variance	Dependent Variable	Fixed Effects	Random effects	AIC	BIC	LogLikelihood	P Value
nlme	equal	mass	treatment*day	id	437.24	478.23	-205.62	
nlme	unequal	mass	treatment*day	id	441.69	511.06	-198.85	0.139

Table S4.

Output from type III test of fixed effects in SPSS to analyze relationship between fixed effects (day, treatment, day*treatment) and dependent variable (viremia), accounting for random effects (id).

Software	Dependent Variable	Fixed Effects	Random Effects	AIC	numerator df	denominator df	F value	P value
SPSS	viremia	day	id	540.466	4	123.594	270.47	0
SPSS	viremia	treatment	id	540.466	1	39.337	0.655	0.423
SPSS	viremia	treatment*day	id	540.466	4	123.594	2.945	0.023

Table S5.

Output from type III test of fixed effects in R studio to analyze relationship between day, treatment, and their interaction on viremia; output is nearly identical between SPSS and R studio, so we were confident that reporting statistics from both software programs would not impact the output.

```
Type III Analysis of Variance Table with Satterthwaite's method
               Sum Sq Mean Sq NumDF   DenDF  F value  Pr(>F)
treatment        0.67   0.670     1  39.336   0.6552 0.42315
day            476.99 119.248     4 123.702 116.6442 < 2e-16 ***
treatment:day   12.00   3.001     4 123.676   2.9351 0.02334 *
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Table S6.

Full statistics from the linear mixed model in SPSS.

Estimates of Fixed Effects[a]

Parameter	Estimate	Std. Error	df	t	Sig.	Interval	
						Lower Bound	Upper Bound
Intercept	1.960458	0.368851	159.805	5.315	0.000	1.232006	2.688911
[treatment =.00]	0.321883	0.525684	157.318	0.612	0.541	-0.716426	1.360191
[treatment =1.00]	0[b]	0					
[time=.00]	-1.960458	0.396255	127.842	-4.947	0.000	-2.744526	-1.176391
[time=2.00]	2.577890	0.400585	128.896	6.435	0.000	1.785318	3.370463
[time=4.00]	5.178810	0.396255	127.842	13.069	0.000	4.394742	5.962877
[time=6.00]	3.203637	0.414423	126.126	7.730	0.000	2.383514	4.023759
[time=10.00]	0[b]	0					
[treatment =.00] * [time=.00]	-0.321883	0.568058	127.781	-0.567	0.572	-1.445902	0.802137
[treatment =.00] * [time=2.00]	-0.147725	0.571087	128.301	-0.259	0.796	-1.277693	0.982243
[treatment =.00] * [time=4.00]	-0.593375	0.570009	127.290	-1.041	0.300	-1.721295	0.534546
[treatment =.00] * [time=6.00]	-1.693626	0.606943	122.947	-2.790	0.006	-2.895037	-0.492214

a. Dependent Variable: PFU.

b. This parameter is set to zero because it is redundant.

Table S7.
Linear mixed model outputs from 'nlme' package in R studio; these values account for repeated measures in the output.

Fixed effects: titer ~ treatment * day					
	Value	Std.Error	DF	t-value	p-value
(Intercept)	0.000000	0.2871384	122	0.000000	1.0000
treatment	0.000000	0.3880181	40	0.000000	1.0000
day2	4.711579	0.3276110	122	14.381628	0.0000
day4	6.868464	0.3332894	122	20.608111	0.0000
day6	3.796707	0.3931153	122	9.657999	0.0000
day10	2.284077	0.4066811	122	5.616384	0.0000
treatment:day2	-0.173625	0.4453719	122	-0.389842	0.6973
treatment:day4	0.270231	0.4469282	122	0.604642	0.5465
treatment:day6	1.369194	0.5118253	122	2.675119	0.0085
treatment:day10	-0.319135	0.5675739	122	-0.562279	0.5750

Table S8.
Parameters used to estimate West Nile virus basic reproductive number. The table provides a definition of each parameter, values used in models, how such values were obtained or estimated (e.g., midpoint and reported range in parentheses), and relevant citations.

Parameter	Definition	Value	Source	Citation
a	Bite rate	0.479/day	Based on 47.9% of *C. quinquefasciatus* females feeding each night	(6)
b	Prob (vector infected by bite)	0.51 (range: 0.17-0.85)	Average proportion of vectors infected when host viremia is in infectious range ($>10^5$)	(7)
c	Prob (host infected by bite)	1	Derived from experiment	N/A
IP (control)	Infectious period (control birds)	2 days	Derived from experiment (days viremia > 10^5)	(8)
IP (ALAN)	Infectious period (ALAN birds)	4 days	Derived from experiment (days viremia > 10^5)	(8)
m	Mosquito mortality rate	1/(25.6 days) (range: 2.93-48.2)	Derived from average adult lifespan of *C. quinquefasciatus*	(9)
k	WNV development rate	1/13 days	Derived from extrinsic incubation period	(10)
M/B	Vector:host ratio	20.16	Derived from vector:host data	(11)

Table S9.
Excel worksheet of DEseq2 results for each contrast.

Table S10.
Excel worksheet of GO results for each of the DEseq2 contrasts.

Table S11.
Excel worksheet of WGCNA results. Included are overall results, module membership for each gene, and GO analyses for modules of interest.

Table S12.
Variance inflation factors (VIF).

	Variables	Tolerance	VIF
	<chr>	<dbl>	<dbl>
1	treatment	0.989	1.01
2	day2	0.247	4.05
3	day4	0.137	7.32
4	day6	0.322	3.10
5	day10	0.677	1.48
6	titer	0.185	5.42

Table S13.
Eigenvalue condition indices used to determine whether collinearity exists; there are no values above 30, therefore, collinearity does not exist between two variables.

	Eigenvalue	Condition Index	intercept	treatment	day2		day4	day6	day10	titer
1	3.30363942	1.000000	1.505112e-02	2.680385e-02	0.004605917	1	0.0036952618	0.0046253228	0.003877121	5.514081e-03
2	1.08014630	1.748860	3.565186e-03	9.220352e-03	0.001881470	2	0.0343264661	0.0121026917	0.269171776	3.627459e-03
3	1.00121935	1.816485	1.063383e-04	5.160158e-04	0.017081323	3	0.0035019340	0.1957529311	0.088235993	1.855106e-07
4	1.00000000	1.817592	0.000000e+00	2.018500e-33	0.112735252	4	0.0186520409	0.0002191967	0.131713136	2.016315e-33
5	0.43460146	2.757089	5.823986e-05	7.079713e-01	0.021598093	5	0.0024236320	0.0399779437	0.095115061	1.803158e-02
6	0.14680278	4.743831	8.943558e-01	2.427874e-01	0.027944388	6	0.0007816947	0.0259071622	0.174303243	6.754369e-02
7	0.03359069	9.917150	8.686336e-02	1.270103e-02	0.814153558	7	0.9366189705	0.7214147517	0.237583671	9.052830e-01

Table S14.
Number of differentially expressed genes classified by DEseq2 at FDR <0.05 and <0.10. In the text, we opted to report the 0.10 values as this is the standard for DEseq2 analysis and the default in the 'DEseq2' R package used to analyze the data (12).

	FDR<0.05	FDR<0.10
Day 2 ALAN vs Control	101	162
Day 6 ALAN vs Control	1989	2775
Control Day 6 vs Day 2	674	997
ALAN Day 6 vs Day 2	2170	2794

Table S15.
Measurements of ALAN at capture sites using VIIRS satellite radiance data from lightpollutionmap.info and handheld lux meters. Handheld measures reported as a range because of the variation in light pollution at a local scale.

Location	Satellite Radiance	Handheld Lux
Lutz	9.53	3.8-4.2
St. Pete Beach	8.35	3.9-4.2

Table S16.
Post-hoc analyses using 'emmeans' in R studio for linear mixed models accounting for repeated measures.

Dependent Variable	Day	Contrast	Estimate	SE	DF	T ratio	P value
Viremia	6	ALAN-control	-1.371	0.465	40	-2.948	0.0053
ΔBody mass	6	ALAN-control	3.111	1.097	40	2.836	0.0071

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