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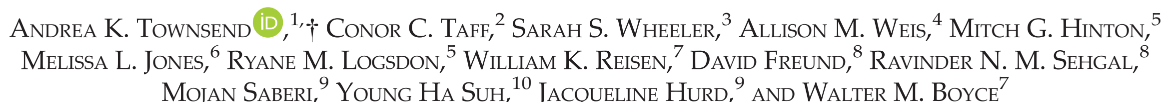
Low heterozygosity is associated with vector-borne disease in crows



Andrea K. Townsend,[1,†] Conor C. Taff,[2] Sarah S. Wheeler,[3] Allison M. Weis,[4] Mitch G. Hinton,[5] Melissa L. Jones,[6] Ryane M. Logsdon,[5] William K. Reisen,[7] David Freund,[8] Ravinder N. M. Sehgal,[8] Mojan Saberi,[9] Young Ha Suh,[10] Jacqueline Hurd,[9] and Walter M. Boyce[7]

[1]*Department of Biology, Hamilton College, Clinton, New York 13323 USA*
[2]*Cornell University Laboratory of Ornithology, Ithaca, New York 14850 USA*
[3]*Sacramento-Yolo Mosquito and Vector Control District, Elk Grove, California 95624 USA*
[4]*Department of Population Health and Reproduction, School of Veterinary Medicine, 100K Pathogen Genome Project, University of California, Davis, California 95616 USA*
[5]*Animal Behavior Graduate Group, University of California, Davis, California 95616 USA*
[6]*Avian Sciences Graduate Group, University of California, Davis, California 95616 USA*
[7]*Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine, University of California, Davis, California 95616 USA*
[8]*Department of Biology, San Francisco State University, San Francisco, California 94132 USA*
[9]*Department of Wildlife, Fish, and Conservation Biology, University of California, Davis, California 95616 USA*
[10]*Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, New York 14853 USA*

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Abstract. Infectious diseases can have devastating impacts on wildlife populations and are of particular concern for small, inbred populations. Identifying specific pathogens that are linked to morbidity and mortality in inbred individuals is a priority for the conservation of small populations, but opportunities to examine them in the wild are rare. Here, we examined the relationship between heterozygosity and infectious disease in American crows (*Corvus brachyrhynchos*), a species that engages in close inbreeding, focusing on three pathogens common in Davis, California, USA: West Nile virus (WNV), *Plasmodium* spp. (avian malaria), and *Campylobacter jejuni*. We found that low heterozygosity at a panel of 33 microsatellite loci was associated with two vector-borne infectious diseases (WNV and avian malaria), but not with infection by the bacterial gut pathogen *C. jejuni*. Reasons for this association with vector-borne pathogens are unclear, but might include behavioral factors and immunological differences associated with inbreeding. Overall, these data are consistent with the idea that inbred individuals may be more susceptible to both novel and endemic vector-borne pathogens, underscoring the importance of protecting genetic diversity within populations and buffering small populations against infectious diseases.

Key words: *Campylobacter jejuni; Corvus brachyrhynchos;* emerging infectious disease; endemic disease; heterozygosity–fitness correlations; inbreeding; inbreeding depression; *Plasmodium;* vector-borne disease; West Nile virus.

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† **E-mail:** aktownse@hamilton.edu

Introduction

Inbreeding depression—the decline in fitness of inbred progeny relative to outbred progeny—may be driven, in part, by increased susceptibility of inbred individuals to infectious disease (Spielman et al. 2004, Eastwood et al. 2017). In theory, inbreeding could elevate infection risk if individuals with low heterozygosity at loci associated with immune responses are able to recognize fewer pathogens than more heterozygous individuals (heterozygote advantage; Radwan et al. 2010, Spurgin and Richardson 2010). Furthermore, inbred individuals might be more likely to succumb to infections if they are physiologically compromised because of the expression of deleterious recessive alleles (Coltman et al. 1999, Luong et al. 2007). Empirically, links between disease and reduced heterozygosity have been documented across a range of taxa and settings, including in wild animal populations (Coltman et al. 1999, Acevedo-Whitehouse et al. 2003), humans (Lyons et al. 2009), captive animals (Ross-Gillespie et al. 2007), and experimental trials (Spielman et al. 2004, Hawley et al. 2005, Smallbone et al. 2016). However, links between heterozygosity and infectious disease have not been detected in all systems or studies, indicating that they may be context-dependent and vary among pathogens (Rosengaus and Traniello 1993, Ortego et al. 2007*b*, Hoeck and Keller 2012).

Empirical data that document the susceptibility of inbred individuals to specific pathogens in wild populations are scarce because of the difficulty of measuring both inbreeding and disease (Daszak et al. 2003, Smith et al. 2009). Nevertheless, concern about potential effects of pathogens (particularly emerging infectious disease) on the persistence of endangered, inbred populations has prompted aggressive conservation actions, including vaccination campaigns, creation of buffer zones, and translocations (Smith et al. 2009, Morrison et al. 2011). A better understanding of the sensitivity of inbred hosts to different pathogens and the characteristics of high-risk pathogens are therefore considered major priorities for conservation (Altizer et al. 2003, Smith et al. 2009).

Here, we examined the relationship between heterozygosity in American crows (*Corvus brachyrhynchos*; "crows" hereafter) and three of their most commonly documented pathogens in Davis, California: (1) West Nile virus (WNV), an emerging, mosquito-borne flavivirus associated with high mortality rates in crows (Yaremych et al. 2004, Reisen et al. 2006, LaDeau et al. 2007); (2) *Plasmodium* spp., a widespread mosquito-borne avian protozoal parasite that has been linked to anemia, poor condition, and lower survival in crows (Townsend et al. 2018*a*); and (3) *Campylobacter jejuni*, a common and widespread bacterial pathogen with a fecal–oral transmission route (Waldenström and Griekspoor 2014, Weis et al. 2014) that has been linked to poor condition and lower survival in crows (Taff and Townsend 2017). Although other parasites have been described in the Davis crow population, we did not evaluate them because they were either low in prevalence (e.g., poxviral dermatitis; Wheeler et al. 2014) or they had no detectable negative health consequences on their hosts (e.g., other haemosporidian parasites; Townsend et al. 2018*a*) and therefore were less likely to exert evolutionary pressure on host genetic diversity and composition (Ferrer et al. 2014). Crows are well suited to this study because they inbreed regularly in some populations (Townsend et al. 2009*a*, 2010*a*), and because low heterozygosity in crows has been associated with elevated disease probability (Townsend et al. 2009*a*), suppressed immune response (Townsend et al. 2010*b*), and lower survival (Townsend et al. 2009*a*, 2010*a*). However, because previous studies combined all infections into a single categorical variable (i.e., disease; Townsend et al. 2009*a*, 2010*b*), links between specific pathogens and inbreeding in crows were unclear. Our results show that low heterozygosity was associated with WNV and *Plasmodium* (the two vector-borne infectious diseases) but not with *C. jejuni*. We interpret these results in light of host resistance, endemicity, and the different modes of transmission of each pathogen.

Materials and Methods

Field site and system

From May 2012 through July 2015, we banded, sampled, and monitored crows in Davis, California. The study area encompassed the University

of California, Davis campus and the adjacent agricultural land (Fig. 1; described in Townsend and Barker 2014, Hinton et al. 2015). The study population included both resident birds, which nested from approximately April–July and remained on or near their breeding territories year-round, as well as adults (including both residents and migrants) captured between January and March adjacent to a winter roost (Hinton et al. 2015, Taff et al. 2016).

We collected infection and heterozygosity data from both adults and nestling birds (Table 1). Adults (n = 58) were captured adjacent to a >7000-bird communal roost, composed of both migrants and residents (Hinton et al. 2015, Townsend et al. 2018*b*), during the winter roosting periods (January to March of 2014 and 2015) using a net launcher (Coda Enterprises) or drop-in trap (Taff et al. 2016). The trap was baited and set before dawn so that crows would encounter it when leaving the roost. Adults were sampled, marked with a numbered USGS band and a unique color band, and released. Nestlings, which were produced by year-round residents, were sampled during the breeding seasons of 2012, 2013, and 2014 (Townsend and Barker 2014, Taff and Townsend 2017). Nestlings (n = 197 birds from 96 nests) were sampled 10–40 d after hatching (mean ± SE = 23.4 ± 0.4 d), either within their nests or as young fledglings on branches immediately adjacent to the nests. Nests were situated on lateral tree branches and accessed by boom lift. Surviving nestlings that were sampled <18 d after hatching were resampled >22 d after hatching. Crows <18 d old were individually marked with a unique toenail clip;

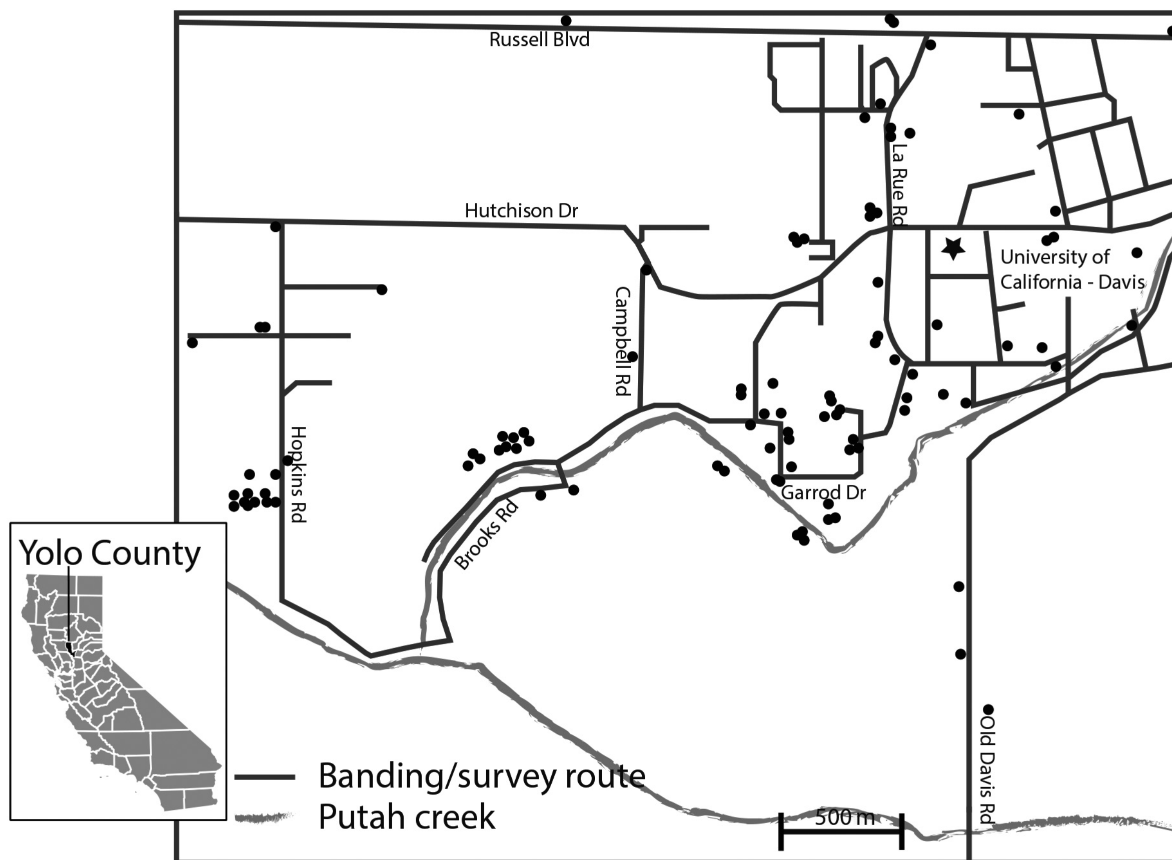

Fig. 1. Map of study site. Sampled nests are indicated by black circles. The standard survey route included all roads drawn. Carcasses were collected along the survey route 1–7 d per week. The star indicates the location of the winter communal roost.

crows >18 d old were marked with both a numbered USGS band and a unique color band. When nestlings were sampled twice during the nestling period, only data from the second sampling point were analyzed, although the results were not qualitatively different when all data were analyzed. Nestling age (± ~3 d) was estimated from approximate hatch date (inferred from the shifting and probing behavior of incubating females, as well as size and feather development of nestlings) following criteria used in Townsend et al. (2010*b*). After their initial marking, we monitored marked birds for survival 1–7 times per week along established census routes (Fig. 1; Townsend and Barker 2014, Wheeler et al. 2014, Hinton et al. 2015, Taff et al. 2016).

Blood samples from live birds were taken from the jugular vein using 27 gauge ½-inch needles and 1-mL syringes. Approximately 400 μL of blood was placed in serum separator tubes for WNV antibody testing. Another drop was placed in Queen's lysis buffer until extraction for genetic analysis. Carcasses of dead crows (n = 86; including both unmarked birds [n = 52] and banded birds [n = 34]), found under nests or during subsequent monitoring, were collected for WNV testing. A tissue sample from each carcass was preserved in lysis buffer for genetic analysis.

Pathogen testing

We banded, genetically sampled, and monitored a total of 255 birds (58 adults; 197 nestlings). Subsets of these birds were screened for each pathogen. Sample sizes for each pathogen test by age class are summarized in Table 1.

Table 1. Sample sizes of birds used in comparisons of homozygosity by loci (HL) with infection status by *Plasmodium*, *Campylobacter jejuni*, and West Nile virus.

Pathogen	n (nestlings/adults)	% positive (nestlings/adults)
Plasmodium	178/58	30/47%
C. jejuni	92/39	75/54%
WNV†	47/76	55/24%

† Birds were classified as "WNV positive" when their carcasses tested positive. Birds were classified as "WNV negative" when they were banded and sampled as adults or nestlings and survived to the endpoint of the study. Although all birds within the "WNV-negative" group tested negative for WNV antibodies at the time of banding, it is possible that they were infected (and survived) after banding.

Plasmodium.—Nestling and adult birds were tested for *Plasmodium* prevalence (presence/absence; Table 1) using molecular screening. Molecular scoring of *Plasmodium* followed methods and modified PCR conditions described in Freund et al. (2016). In brief, genomic DNA was screened for *Plasmodium* using a nested PCR (Hellgren et al. 2004). We identified *Plasmodium* in samples with parasite cyt *b* amplification by sequencing the fragments bidirectionally using the BigDye version 1.1 sequencing kit (Applied Biosystems, Foster City, California, USA) on an ABI PRISM3100 automated sequencer (Applied Biosystems). Sequences were edited in Sequencher 4.9 (GeneCodes, Ann Arbor, Michigan, USA), aligned by eye, and then subjected to a BLAST search to ensure that *Plasmodium* was amplified. Although this assay also screened for two other haemosporidian parasites (*Leucocytozoon* and *Haemoproteus*), the current study was focused on confirmed pathogens, and—unlike *Plasmodium*—*Leucocytozoon* and *Haemoproteus* did not have detectable negative health consequences in crows (Townsend et al. 2018*a*). However, for the purpose of comparison with other studies that have evaluated these genera, we present their relationship with heterozygosity in Appendix S1: Fig. S1.

Campylobacter jejuni.—To test birds for *C. jejuni* (Table 1), we collected fresh fecal samples from individual plastic carriers in which we held the crows until banding. Fecal samples were placed in Amie's clear gel transport media (Remel BactiSwab; Thermo Fisher Scientific, Waltham, Massachusetts, USA) and stored in a cooler on ice until submission to the UC Davis Veterinary Medicine Teaching Hospital for *C. jejuni* culturing and biochemical testing within six hours of collection (testing procedures described in detail in Weis et al. 2014). Fecal samples were collected from all adults but only opportunistically from nestlings (Taff et al. 2016) because not all nestlings defecated during handling. Carriers were disinfected with a 10% bleach solution between birds to prevent cross-contamination of samples.

West Nile virus.—To examine the association between heterozygosity and WNV infection (Table 1), live birds were tested for WNV antibodies at time of banding using a WNV-neutralizing

antibody by plaque reduction neutralization test (PRNT). Dead birds found along the established survey route (Fig. 1; Wheeler et al. 2014, Hinton et al. 2015) were tested for the presence of the virus. To test for presence of antibodies, sera were challenged with 100 plaque-forming units at a final serum dilution of 1:20 using standard PRNT methods with a 90% reduction in virus, described in Wheeler et al. (2012*b*). To test for presence of virus, we extracted WNV RNA from oral swabs or kidney samples (which have similar accuracy in detecting WNV in corvids; Padgett et al. 2006) by Magmax using Life Technologies protocols. We used qRT–PCR with primers and probe specific for the envelope region of the WNV genome (Lanciotti et al. 2000), a ViiA 7 Real-Time PCR system (Life Technologies), and SensiFAST One-Step reagents (Bioline, Taunton, MA; testing procedures described in Wheeler et al. 2014, Hinton et al. 2015). Dead birds were classified as WNV positive when their threshold cycle (C_t) scores were <40. We excluded birds with deaths attributable to unknown, traumatic, or non-WNV disease processes (Wheeler et al. 2014) from consideration because sample sizes of other disease processes were too small for meaningful analysis or cause of death was ambiguous.

Heterozygosity analyses

We genotyped all birds at a panel of 33 microsatellite loci developed for American crows. Locus characteristics (e.g., alleles/locus, null allele frequencies, and tests of Hardy-Weinberg equilibrium) and PCR conditions are given in the supplementary materials (Appendix S2: Tables S1–S2). Mean allelic diversity was 9.8 ± 1.2 alleles/locus (range: 2–39 alleles/locus), and frequencies of inferred null alleles were <0.1 at all loci (mean null allele frequency: 0.03 ± 0.005). Six of 33 loci deviated significantly from Hardy-Weinberg equilibrium (i.e., homozygosity excess); such departures are consistent with the occurrence of inbreeding in other natural populations (e.g., Rougeron et al. 2009, Elias et al. 2010). We scored all birds at a minimum of 30 loci; most were scored at the complete panel of 33 loci (mean proportion of loci scored >0.99).

We used this panel to estimate homozygosity by loci (HL), an index of multilocus heterozygosity weighted by allelic variation at each locus (Aparicio et al. 2006) using extension Rhh (Alho et al. 2010) in program R v3.3.1 (R Core Team 2016). We used HL because it does not require specification of background allele frequencies, which were inestimable for migratory birds. Previous work comparing heterozygosity at a subset of markers in this panel with pedigree information, parental relatedness coefficients, and heterozygosity–heterozygosity correlations has shown that it is an accurate index of parental relatedness and inbreeding in crows (Townsend et al. 2009*a*, *b*, 2010*a*). Moreover, genome-wide and multilocus heterozygosity (identity disequilibrium) at this panel of markers, estimated by the g_2 statistic using the software RMES (10,000 permutations; David et al. 2007), were significantly correlated in the Davis, California population ($g_2 = 0.002 \pm 0.001$ SD, $P = 0.02$), indicating that inbreeding was occurring with variation sufficient to detect heterozygosity–fitness correlations (Szulkin et al. 2010).

Statistical analyses

We examined the effects of HL on *Plasmodium* and *Campylobacter* prevalence (presence/absence) in nestlings in generalized linear mixed models (glmer; binomial distribution; Laplace approximation) in R package lme4 (Bates et al. 2015), with *Plasmodium* or *Campylobacter* (yes/no) as the response, HL and age as fixed effects, and nest as a random effect to account for non-independence among brood mates. We examined prevalence in adults in generalized linear models (glm; binomial distribution), with *Plasmodium* or *Campylobacter* as the response and HL as the predictor. We examined the effects of HL on WNV in a glm (binomial distribution), with WNV as the response and HL and cohort (initially banded as adult or nestling) as the predictors. We analyzed WNV in a single model (rather than as separate models for nestlings and adults) because most of the WNV-positive birds that were banded as nestlings died months after fledging (i.e., as subadults or adults; see *Results*). In contrast, we analyzed *Plasmodium* or *Campylobacter* in separate adult and nestling models because these analyses referred to infection status at the time of sampling (i.e., in the nest for the nestling cohort).

Results

Heterozygosity and Plasmodium

We scored 236 birds for *Plasmodium* infection. Among adults, 47% (27/58) screened positive, whereas 30% (53/178) of nestlings screened positive (Table 1). Four different cytochrome *b* lineages of *Plasmodium* were identified among the birds that tested positive (corresponding to sequences named TROAED24 [36%], SEIAUR01 [32%], LAIRI01 [27%], and MOLATE01 [5%] in the MalAvi Database for Avian Haemosporidian Parasites; Bensch et al. 2009). We did not analyze them separately because of small sample sizes of each lineage. *Plasmodium* infection was linked to low heterozygosity in nestlings but not in adults (Fig. 2A). The likelihood of nestlings testing positive for *Plasmodium* increased with HL ($\beta(\log(HL)) = 5.40 \pm 2.63$; $z_{(174)} = 2.01$; $P = 0.04$; $n = 178$ birds from 68 families): The odds of testing positive were 17.2 times higher for the most homozygous birds across the range of HL values. The odds also increased with nestling age ($z_{(174)} = 2.01$; $P = 0.03$). In contrast, HL was not associated with *Plasmodium* prevalence in adults ($z_{(56)} = 0.81$; $P = 0.42$; $n = 58$ adults).

Heterozygosity and *C. jejuni*

Of the 131 birds tested for *Campylobacter jejuni*, 90 (68.7%) tested positive (i.e., were shedding *C. jejuni* in their feces; Table 1). Thirty-nine of these birds were sampled as adults (53.8% of which tested positive), and 92 were sampled as nestlings (75% positive). Infection by *C. jejuni* was not associated with heterozygosity (Fig. 2B): *C. jejuni* infection was not related to HL in nestlings ($z_{(89)} = -1.1$; $P = 0.28$; $n = 92$ nestlings from 49 family groups) or adults ($z_{(37)} = -0.2$; $P = 0.87$; $n = 39$ adults).

Heterozygosity and WNV

To examine the association between heterozygosity and WNV infection, we compared the HL scores of the 44 birds that died and tested positive for WNV (hereafter "WNV-positive" birds; 44/86 total carcasses collected) with the HL scores from the 79 banded birds that were detected alive at the endpoint of the study (hereafter "WNV-negative" birds). Forty-two of the 86 carcasses that we collected tested negative for WNV, with cause of death from unknown, traumatic, or non-WNV disease processes (Wheeler et al. 2014); as stated above, we excluded these birds from consideration because the sample sizes of other fates were too small for meaningful analysis or cause of death was ambiguous. Twenty-six of the 44 WNV-positive birds had been banded in the nest and subsequently died WNV positive. Only two of these birds died within the nest; the other 24 birds fledged and died within 1–15 months (mean: 3.75 ± 1.03 months) after fledging from the nest. The remaining 18 WNV-positive birds were unbanded adults that were collected along the survey route and from beneath the >7000-bird winter communal roost.

The WNV-negative group was composed of 58 birds initially banded as adults, as well as 21 birds initially banded as nestlings that were still detected alive at the endpoint of the study (July 2015; surviving >15–39 months post-hatching, depending on hatch year). All of the 58 adults and 197 nestlings tested in this study were negative for WNV antibodies at the time of banding (including the 58 birds in the WNV-negative group), indicating no previous WNV infection at that time. It is possible, however, that the birds in the WNV-negative group were infected (and survived) after banding. Therefore, the WNV-negative category could have included both birds that had never been infected and those that survived after infection.

The WNV-positive and WNV-negative categories each included two cohorts of birds (Fig. 2C), based on time when initially banded or sampled: (1) birds that were initially banded as nestlings that eventually died and tested positive for WNV ($n = 26$; the WNV-positive nestling cohort) and birds that were banded as nestlings that survived to the endpoint of the study ($n = 21$; the WNV-negative nestling cohort) and (2) carcasses of unbanded adults, collected in the fall and winter months (including both resident and migratory birds), that tested positive for WNV ($n = 18$; WNV-positive adult cohort) and live adults that were captured and banded in winter months (again including both resident and migratory birds) that survived to the endpoint of the study ($n = 58$; WNV-negative adult cohort).

Although the nestling and adult categories differed in both age at time of banding and source

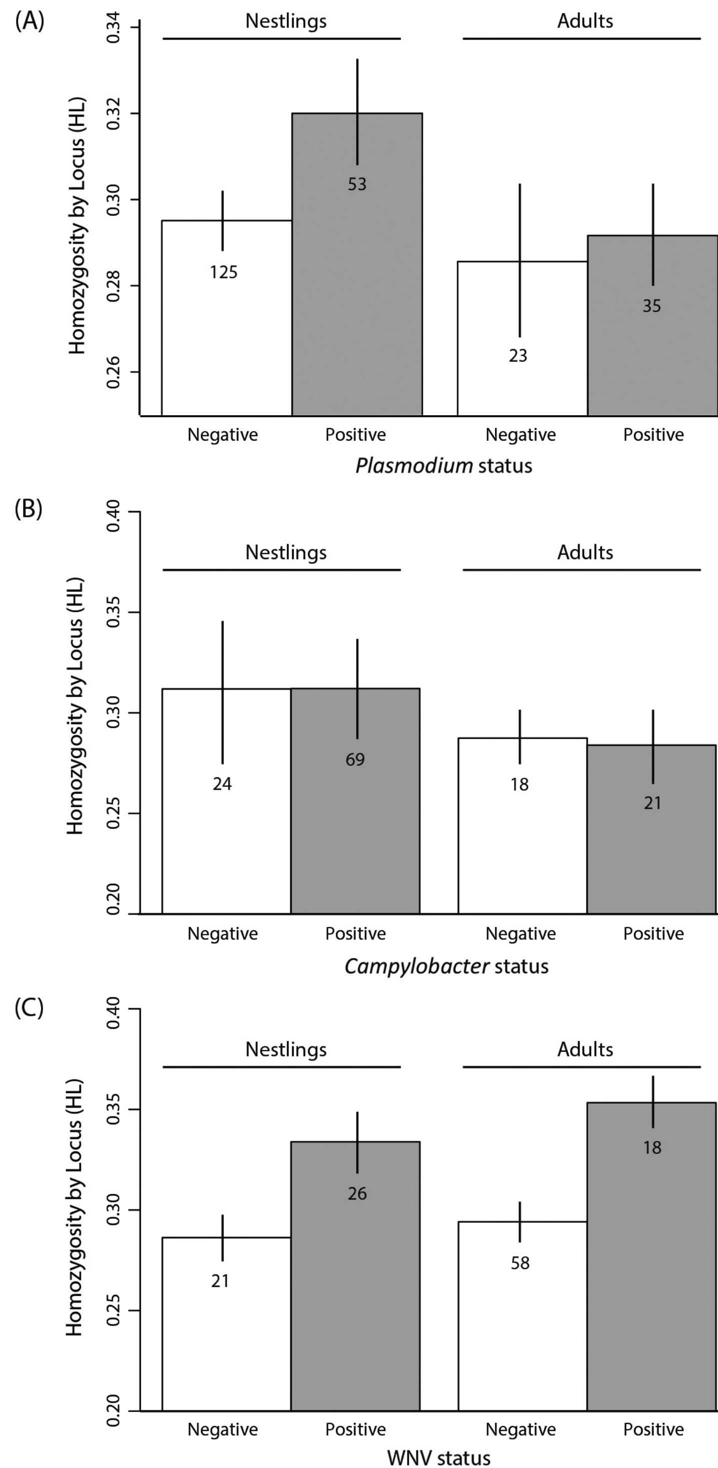

Fig. 2. Mean (± SE) homozygosity by locus (HL) score with age class (e.g., time of initial sampling) and with (A) *Plasmodium* infection status, (B) *Campylobacter jejuni* infection status, and (C) WNV infection status. Analytical and classification details are given in text. Increasing HL scores indicate increasing homozygosity. Numbers within bars refer to sample sizes.

population, patterns of HL with WNV infection were consistent across cohorts (Fig. 2C). There was a positive association between WNV infection and HL (β(log(HL)) = 9.33 ± 2.98; $z_{(120)}$ = 3.1; $P = 0.002$; $n = 123$ birds): The odds of a WNV-positive classification were 83 times higher for the most homozygous birds across the range of HL values. The odds of a WNV-positive classification were also higher among birds first sampled as nestlings than as adults ($z_{(120)} = 3.54$; $P < 0.001$).

Coinfections

There were no detectable correlations in infection probability among pathogens. Both *Plasmodium* status and *C. jejuni* status were known for 111 birds (n = 38 adults; 83 nestlings). In a generalized linear model (binomial distribution) with *Plasmodium* infection (0/1) as the response and *C. jejuni* infection and cohort (adult/nestling) as predictors, infection by *C. jejuni* was not a significant predictor of *Plasmodium* infection ($z_{(108)} = -1.2$; $P = 0.22$), although *Plasmodium* prevalence was significantly higher among adults than nestlings ($z_{(108)} = 3.3$; $P < 0.001$). The status of all three pathogens (*Plasmodium*, *C. jejuni*, and WNV) was known for 25 birds, all of which were initially sampled as nestlings. Among these 25 birds, neither *Plasmodium* ($z_{(22)} = 0.67$; $P = 0.5$) nor *C. jejuni* ($z_{(22)} = 0.62$; $P = 0.5$) infections were significant predictors of subsequent WNV infection (glm with WNV status (0/1) as the response and *Plasmodium* infection and *C. jejuni* infection as predictors).

Discussion

Identifying specific pathogens that are linked to morbidity and mortality in inbred individuals and the characteristics of these high-risk pathogens are priorities for the conservation of small populations (Altizer et al. 2003, Smith et al. 2009). In the current study, we found that low multilocus heterozygosity—which previous work has shown to correlate with inbreeding in crows (Townsend et al. 2009*a*, 2010*b*)—was associated with two vector-borne infectious diseases: an emerging epidemic pathogen (WNV) and an endemic, common one (*Plasmodium* spp). Specifically, the odds of an active *Plasmodium* infection while in the nest were 17.2 times higher, and the odds of eventually dying with a WNV infection were 83 times higher, for the most homozygous birds across the range of HL values. We found no associations between heterozygosity and infection by *Campylobacter jejuni,* a common and widespread bacterial pathogen with a fecal–oral transmission route. Overall, our results support the widely held hypothesis that inbred individuals may be more susceptible to certain infections than outbred individuals (Altizer et al. 2003, Spielman et al. 2004, Eastwood et al. 2017), and provides empirical data—rare in wild, free-living populations—on how this susceptibility varies among specific pathogens.

Both of the pathogens associated with low heterozygosity in crows (*Plasmodium* and WNV) are mosquito-borne. The factors mediating this link are unknown, but might include aspects of host behavior. For example, individual hosts might be able to reduce infection risk from mosquito-borne pathogens through differential site selection (e.g., establishing territories with fewer mosquito vectors) and through specific motor sequences that repel vectors (e.g., preening, wing flicking; Darbro and Harrington 2007, Ugelvig et al. 2010). These energetically taxing antiparasite behaviors may be more challenging for inbred crows, which tend to be in poor condition relative to outbred crows (Townsend et al. 2010*b*). Indeed, data from other taxa indicate that territory acquisition (Potts et al. 1994) and antiparasite behaviors (Luong et al. 2007, Ortego et al. 2007*a*, Ugelvig et al. 2010) are suppressed among inbred individuals. In contrast, these behavioral defenses might be less applicable to pathogens with a fecal–oral route of transmission, such as *C. jejuni*, where strains tend to be passed among family members: Nestlings within the same brood tend to be either universally infected or uninfected with *C. jejuni*, and strains are more similar among nest mates than expected by chance (Taff et al. 2016). We note that even though host heterozygosity was not associated with elevated risk of infection by *C. jejuni*, inbreeding could still exacerbate the negative consequences of *Campylobacter* infection (Taff and Townsend 2017) through its negative effects on overall body condition (Townsend et al. 2010*b*).

Compromised immune defenses could also play a role in the elevated likelihood of vector-borne

disease in inbred crows. Studies in other avian species indicate that immunological defenses can operate in combination with behavioral defenses to protect hosts against vector-borne pathogens (Waite et al. 2014). In crows, inbreeding does appear to be associated with a suppressed or altered immune response. Previous work has shown that the blood of less heterozygous crows killed fewer bacteria in a microbicidal assay (reflecting the ability of the host to stop a potential pathogen; Millet et al. 2007) than more heterozygous crows (Townsend et al. 2010*a*, *b*). However, the hypothesis that a suppressed immune response among inbred birds increases their infection risk assumes that crows can mount an effective immune response against *Plasmodium* and WNV. Some avian taxa in North America do appear to mount a protective immune response against *Plasmodium*, as evidenced by specific alleles associated with resistance (Loiseau et al. 2011, Sepil et al. 2013), combined with apparent local adaptation to sympatric strains (Sarquis-Adamson and MacDougall-Shackleton 2016). Likewise, we found some evidence for an adaptive immune response to *Plasmodium* infection among crows in the Davis, California population: Globulins (the fraction of the plasma protein that contains parasite-specific antibodies) were elevated in *Plasmodium*-infected crows (Townsend et al. 2018*a*).

In contrast, however, the extent to which crows are able to mount an effective immune response to WNV is unclear (Nemeth et al. 2011, Staley and Bonneaud 2015). Early reports documented 100% mortality of crows after experimental infection (Brault et al. 2004), although reports of antibodies in field-collected crows indicated that they might have resistance in some populations (Wilcox et al. 2007, Reed et al. 2009). In our study, however, no crows tested positive for WNV antibodies at the time of banding, indicating no previous WNV infection at that time. If crows were surviving after WNV infection in this population, we would expect to find antibodies among adult birds, although our sample of adults (n = 58) was small. Sample size of nestlings was larger (n = 197), yet the lack of WNV antibodies among nestlings was less surprising because the WNV season was in its early stages while these birds were in the nest: Only 2 of 197 birds died of WNV while still in the nest. Therefore, their likelihood of exposure (and exhibiting a detectable antibody response; Nemeth et al. 2011) was small at the time of sampling. Moreover, maternal inheritance of WNV antibodies in other passerines appears to be short-lived (i.e., undetectable more than nine days post-hatch; Perez-Ramirez et al. 2014). Therefore, nestling crows may have been sampled too early in the season to detect responses to a current infection and too late to detect maternal antibodies (if present).

Although we did not detect antibodies, circumstantial evidence suggests that some crows tolerated long-term, persistent infections in our Davis, California population. Hinton et al. (2015) documented continuous crow mortality associated with WNV throughout the winter months, when mosquitoes were quiescent and unlikely to transmit the virus. Moreover, viral concentrations were significantly lower—which could indicate persistent infection (Wheeler et al. 2012*a*, Reisen et al. 2013)—among WNV-positive birds in winter compared to summer. In addition, many of the crows that died with WNV in winter showed signs of other infections (e.g., bacterial, fungal, viral, and protozoal; Wheeler et al. 2014). It is possible that crows were surviving despite persistent, prolonged WNV infection but succumbed to multiple infections during the stressful winter months and that this likelihood of mortality was elevated for inbred birds. A similar combination of infection by sublethal parasites and harsh winters led to elevated mortality among inbred Soay sheep (Coltman et al. 1999). In this case, the link that we documented between low heterozygosity and WNV could reflect an elevated likelihood that an inbred bird will die after infection, rather than an elevated likelihood of acquiring an infection.

The relationship between heterozygosity and *Plasmodium* was observed only among birds sampled as nestlings, not in birds sampled as adults. These results are similar to other studies, with adult birds as hosts, that found no correlation (Ortego et al. 2007*b*) or even detected a negative quadratic relationship (Ferrer et al. 2014) between host heterozygosity and *Plasmodium* infection. In our study, adult *Plasmodium* infections were likely chronic rather than acute, because we sampled them in winter months, when mosquitoes were quiescent. The correlation

between heterozygosity and *Plasmodium* infection could diminish among chronically infected adults if, for example, inbred birds were less likely to survive initial, acute infection (e.g., because they were in poor condition; Townsend et al. 2010*b*) than outbred birds. If this was the case, the sample of chronically infected adults would be enriched with relatively outbred birds. However, without longitudinal infection data from the same individuals, we cannot determine the processes that resulted in the lack of correlation among adults, because variation in the time of exposure along with differential survival or clearance can all contribute to patterns of *Plasmodium* infection (Freeman-Gallant and Taff 2017).

Understanding the relationship between inbreeding and disease is critical to the conservation of small or fragmented populations, in which the probability of inbreeding is elevated (Pertoldi et al. 2007, Smith et al. 2009, Brzeski et al. 2015). Our results illustrate the elevated risk that both endemic and emerging vector-borne diseases (WNV and—in nestlings—*Plasmodium*) can pose for inbred individuals and populations, underscoring the need to buffer small populations against infectious disease. We note, however, that the mechanism underpinning this association (e.g., heterozygote advantage, the effect of specific alleles; Spurgin and Richardson 2010) is unknown. Moreover, we only report data from a single population, and interactions between pathogens and hosts can vary among populations, potentially in ways that will affect the relationship between disease risk and inbreeding. For example, the extent to which inbreeding might increase the home field advantage by preserving local resistance alleles (Sarquis-Adamson and MacDougall-Shackleton 2016) might be affected by factors such as virulence of the local *Plasmodium* species and the occurrence of alleles with antagonistic effects (e.g., positive effects in some populations and negative effects in others; Loiseau et al. 2011). Likewise, the ability for crows to resist and recover from a specific pathogen could vary among populations and change over time; indeed, small changes (i.e., single substitutions) in the genotype of WNV have led to large changes in virulence in crows (Brault et al. 2007). The costs and nature of resistance to a specific pathogen could also change because of other pathogens encountered within a host's environment (Koskella et al. 2011). A more complete understanding of links between inbreeding and pathogen risk will require consideration of additional populations and pathogens that vary in characteristics such as endemicity, mode of transmission, and virulence.

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Supporting Information

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.2407/full