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Effects of Drift, Selection and Gene Flow on Immune Genes in Prairie Grouse

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EFFECTS OF DRIFT, SELECTION AND GENE FLOW ON IMMUNE GENES IN PRAIRIE

GROUSE

by

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ABSTRACT

EFFECTS OF DRIFT, SELECTION AND GENE FLOW ON IMMUNE GENES IN PRAIRIE
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Fragmentation of natural habitats is related to population decline in many species. The resulting

small and isolated populations are expected to lose genetic variation at a rapid rate, which

reduces the ability to adapt to environmental change. One concern is that small populations are

more susceptible to emerging pathogens due to the loss of variation at immune genes. My

dissertation examined the relative effects of gene flow, genetic drift and selection on immune

genes in prairie-chickens (*Tympanuchus cupido*), a species that has undergone drastic population

declines across their range. In the first chapter, I examined how artificial gene flow through

translocations of birds from Minnesota to the threatened Wisconsin population influenced

genetic diversity at both neutral loci and immune genes. My second chapter explored how

selection and drift shaped variation at two different functional categories of immune genes across

prairie-chicken populations, including the critically endangered Attwater's prairie-chicken (*T.c.*

attwateri). My third chapter assessed how immune gene variation in captive-bred Attwater's

prairie-chickens is related to their immune response and survival in the wild. Overall, this

dissertation provides a better understanding of how evolutionary mechanisms are shaping

variation at immune genes in threatened species at both the population and individual level.

TABLE OF CONTENTS

Chapter 3 – Specific alleles at immune genes, rather than genome-wide heterozygosity, are related to immunity and survival in the critically endangered Attwater's prairie-chicken

LIST OF FIGURES

LIST OF FIGURES (SUPPLEMENTARY)

LIST OF TABLES

ACKNOWLEDGEMENTS

CHAPTER 1

Genetic Restoration of a Threatened Population of Greater Prairie-Chickens

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Abstract

Supplemental translocations to small, isolated populations can be a valuable strategy to counteract the effects of genetic drift by increasing genetic diversity. We studied the genetic consequences of a translocation of greater prairie-chickens (*Tympanuchus cupido pinnatus*) to Wisconsin, which has a small population (<550 birds) with low genetic diversity. During 2006-2009, 110 females were translocated to Wisconsin from Minnesota, which has greater genetic diversity. Two years after the final translocation, we detected introgression of unique Minnesota alleles. Although there was an increase in mtDNA diversity to near historic levels, there was no change in diversity at microsatellites or the major histocompatibility complex (MHC). Computer simulations of drift predicted that microsatellite diversity would have been lower in the absence of the translocation, and, thus, the translocation was a success in temporarily stemming the ongoing erosion of genetic variation through drift. Overall, our results caution that introgression of new alleles varies for genetic markers that differ in selection and inheritance, and, thus, the success of genetic restoration projects may depend on how the goals are defined.

Introduction

Many species that once inhabited large continuous landscapes are now forced to exist in small isolated populations whose long-term persistence is uncertain. Supplemental translocation or restocking is being used increasingly to reduce the risk of extinction from both demographic and genetic stochasticity (reviewed by Champagnon et al., 2012; Perez et al., 2012). Some supplemental translocations have resulted in greater reproductive success or population growth following translocation, but claims for genetic "rescue" or "restoration" are often controversial, because it is not always clear if the population response was due to genetic or environmental changes (Adams et al., 2011). Genetic analysis of translocated individuals is important in this respect (Table 1.1), because it allows researchers to ascribe population responses directly to changes in genetic diversity from the translocation, rather than natural processes such as mutation or immigration.

Another issue with genetic studies of translocations is that they often estimate levels of variation using adaptively neutral markers, which may not reflect variation at functional genes that are important to population fitness. In particular, variation at immunity genes is of increasing concern as previously unknown diseases in wildlife, such as amphibian chytridiomycosis and Tasmanian devil facial tumor disease, have emerged as serious threats to populations. In these cases (Siddle et al., 2007; Savage and Zamudio, 2011) and others, resistance to disease has been linked to genes of the major histocompatibility complex (MHC), which code for molecules that recognize pathogens and initiate the adaptive immune response. Similar to neutral diversity, MHC diversity in small isolated populations is typically low, but the impact on population viability is not well understood (Radwan et al., 2010). To date, only one study of snakes has documented the effect of supplemental translocations on MHC genetic diversity (Madsen et al.,

1999).

Here, we assess the consequences of a supplemental translocation on both neutral and MHC genetic variation in a bottlenecked population of greater prairie-chickens (*Tympanuchus cupido pinnatus*) in Wisconsin (WI). Early in the 1900s, there were > 50 000 prairie-chickens in WI and they occupied nearly every county in the state (Grange, 1948). However, during the 1950s the population declined to 1500 birds, and the range contracted to 344 km^2 in central WI (Figure 1.1). Today, the population has declined to 256 displaying males, most of which (122) are in the Buena Vista management area (Wisconsin Department of Natural Resources, WDNR, unpubl. data). The population continues to be isolated as the nearest contemporary populations are over 590 km away in MN and IL. Comparisons of genetic variation before the bottleneck in the 1950s and afterwards (1996-1999) showed that the population has lost genetic variation. Surprisingly, more variation has been lost at functional (44%, MHC) than neutral (8%, microsatellites) markers in the Buena Vista population (Bellinger et al., 2003; Johnson et al., 2004; Eimes et al., 2011).

Based on the isolation and loss of genetic diversity of the WI prairie-chicken population, the WDNR decided to translocate birds from Minnesota (MN) to increase genetic diversity. The population in western MN was chosen as the source of translocated birds, because it had greater genetic diversity than the WI population, similar to larger populations in Kansas and Nebraska (Figure 1.1; Johnson et al., 2003), and it was at a similar latitude, so birds would be acclimated to a more northern environment. The Buena Vista management area was chosen to receive all of the MN birds because it had the most grassland habitat (5140 h) and was intensively managed for prairie-chickens.

In this study, we evaluated whether these translocations from MN were successful at

4

increasing genetic diversity of prairie-chickens on the Buena Vista management area, hereafter referred to as the WI population. We also examined the potential and actual genetic contributions of translocated females from MN, and, thus, the efficiency of introgression at both neutral (microsatellite, mitochondrial DNA) and functional (MHC) genetic markers. Overall, our results caution that introgression of new alleles varies for genetic markers that differ in selection and inheritance, and, thus, interpreting the success of genetic restoration projects may depend on how the goals are defined. Although many studies claim that translocations are successful in restoring genetic variation, at least in the short term (Table 1.1), our study also revealed that drift continues to erode genetic variation, and, thus, translocations may only be a temporary solution for small, isolated populations.

Methods

Pre- and Post-translocation Wisconsin Sample Collection

For the pre- and post-translocation samples, we collected blood and feathers, respectively, for genetic analysis from greater prairie-chickens at the Buena Vista management area, Portage County, WI (44° 20′ 15″, 89° 38′ 49″; Figure 1.1). The WI pre-translocation blood samples ($n = 41$) were from males captured between 1996 and 1999 that were previously studied by Bellinger et al. (2003) and Johnson et al. (2003, 2004). The WI post-translocation samples consisted of feathers collected by the WDNR at 12 leks located throughout the Buena Vista management area during the March-May 2011 breeding season. These post-translocation samples covered the same geographic area as the pre-translocation samples. To extract DNA from the post-translocation WI feathers, we followed the molted feather protocol of Bush et al. (2005) using a Qiagen DNeasy® Tissue Kit (Valencia, California, U.S.A.).

5

Translocated females

Female prairie-chickens (n = 110) from the MN population (Clay, Norman, Polk and Red Lake Counties; Figure 1.1) were captured during August-September 2006-2009 and transported by vehicle or airplane to WI for release. Only females were chosen for translocation because prairie-chickens have a lek mating system in which relatively few males (~10%) breed each year (Robel, 1970), and, thus, translocating females, which would presumably all breed, would be more likely to result in introgression. Although all females released in 2006 (n = 40) and 2009 (n = 19) were sampled for this study, we obtained fewer samples from females released in 2007 (n = 3/24, 13%) and 2008 (n = 24/27, 89%). In total, we obtained blood samples from 78% (86/110) of the translocated females. We extracted DNA from the blood samples using a saturated salt solution method (Miller et al., 1988). All translocated females were radio-tracked following release to determine survival and nesting success (see Hess et al., 2012 for details).

Microsatellites

Our estimates of genetic variation in prairie-chickens were based on seven microsatellite loci originally developed for other galliforms. Details of the microsatellite PCR amplifications are described in Bellinger et al. (2003) and Hess et al. (2012). All samples were analyzed on a capillary-based sequencer (ABI 3730) and sized using STRAND software (Toonen and Hughes, 2001; http://www.vgl.ucdavis.edu/STRand). To ensure consistency in sizing alleles, we re-genotyped the pre-translocation samples that were previously analyzed on a polyacrylamide gel sequencer (ABI 370). We binned allele sizes at all seven loci using the program MSATALLELE (Alberto, 2009).

6

We were able to extract DNA from 112 of the 300 feathers collected. To identify and remove duplicates from the 112 samples, we estimated the probability that any two randomly chosen individuals in a population will share the same genotype (probability of identity) using seven microsatellite loci. This probability is biased downward, so as a more conservative upper bound, we also estimated the probability of identity for siblings (Waits et al., 2001). Both of these probabilities were calculated in GENALEX 6.5 (Peakall and Smouse, 2012). For the post-translocation WI samples, the probability of identity and probability of identity for siblings were low (1.8×10^{-8} and 1.8×10^{-3}, respectively). Based on the probability of identity we found 81 unique genotypes (individuals) among the 112 samples (1-3 repeats per individual), which were used in all subsequent analyses.

We examined the overall repeatability of microsatellite genotyping by using a version of the 'comparative method' proposed by Frantz et al. (2003). Using this approach, we performed additional PCR amplifications and repeated genotyping on 41% (232/567) of the original feather genotypes, sampled across all seven loci. In particular, we genotyped 161 homozygous genotypes three times and found that 19 genotypes differed because of allelic dropout (12%); these genotypes were changed to heterozygotes in the final data set when we observed both alleles in two of the three independent PCRs. We found no discrepancies among 71 heterozygote genotypes that were amplified and sequenced twice (i.e., false alleles; Broquet and Petit, 2004). Standard measures of microsatellite variation (number of alleles, allelic richness, and observed and expected heterozygosity) and population structure (D_{est}; Jost, 2008) were estimated in GENALEX or FSTAT 2.9.3 (Goudet, 1995; Peakall and Smouse, 2012). We used the default settings in the program ARLEQUIN 3.5.1.3 (Excoffier and Lischer, 2010) to assess deviations from Hardy-Weinberg equilibrium (HWE) and evidence of linkage disequilibrium (LD) between pairs of loci

for each population. None of these loci departed from HWE in our previous studies (see also Bellinger et al., 2003 and Hess et al., 2012), but in this study, ADL146 showed a heterozygote deficiency (Table S1.1). There was no evidence of LD in the pre-translocation WI sample, which we used for the simulations.

Sex determination

The sex of individuals in the post-translocation sample was determined by amplifying an intron of the CHD gene (Kahn et al., 1998; see Supporting Information for more details). All individuals ($n = 81$) were identified as males and, thus, no translocated females were included in the post-translocation sample. These 81 males comprised 60% of the male population at the Buena Vista management area in spring 2011 (based on lek counts; WDNR unpubl. data).

Drift simulation

We used the computer program GENELOSS (England and Osler, 2001) to simulate genetic drift in the WI population over the 15 years (~ 7.5 generations) between the pre- (1996) and post- (2011) translocation samples. This method was also used by Bellinger et al. (2003) to simulate the loss of microsatellite variation over the 50 years (~25 generations) between their pre- (1951) and post (1996-1997) bottleneck samples from Buena Vista. We used the simulation to compare levels of genetic variation observed in the post-translocation population with that expected from genetic drift alone (i.e., without translocation). For example, if values of post-translocation genetic diversity were equal to the pre-translocation WI, and greater than the simulated levels, then it would suggest that genetic diversity was maintained in the population. The initial conditions of the simulation were based on microsatellite allele frequencies and effective population size

8

estimates from the Buena Vista sample before the translocation (Bellinger et al., 2003). We used

an N_e estimate of 77 birds which was calculated from the harmonic mean of census estimates as

described by Bellinger et al. (2003). This estimate of N_e was also similar to the midpoint of N_e

estimates (80) for Buena Vista based on historic changes in microsatellite variation (22 to 137

birds, for 20 generations in Table 5 of Johnson et al. 2004). The GENELOSS simulation assumes

non-overlapping generations, a constant Ne and a closed population. Obviously, some of these

assumptions were violated, but the constant Ne estimate used in the simulation is probably

conservative, because the Buena Vista population declined during and after the translocation

despite the addition of 110 females from MN (2007- 2012: 680, 492, 440, 292, 334, 310

estimated birds; WDNR, unpubl. data).

Mitochondrial control region

We sequenced the mitochondrial DNA (mtDNA) control region using primers 16775L (Quinn,

1992) and H774 (Sorenson et al., 1999) following methods in Johnson et al. (2004). Both

forward and reverse sequences for each individual were aligned and edited to produce a 384 bp

consensus sequence in GENEIOUS 5.4 (Biomatters, Aukland, NZ). Both forward and reverse

sequences were identical in all samples. We were unable to amplify nine of the 81 post-

translocation samples, possibly because of the lower quality DNA from feathers. Haplotype (*h*)

and nucleotide diversity (π) were calculated using the program DNASP 4.10.4 (Librado and

Rozas, 2009), and population pairwise θ_{ST} values were estimated using ARLEQUIN.

MHC class I

We examined diversity at exon 3 of MHC class I (see supporting information for methods).

Based on our initial analyses of the pre-translocation sample, we chose a sample size of >25 birds because this number is expected to result in finding all alleles with >90% probability (i.e., the binomial probability of missing one allele = 0.09, assuming 11 equally-frequent alleles). Our final sample size was 30 pre-translocation WI, 31 post-translocation WI, and 26 translocated MN females (from 2006). We used DNASP to calculate haplotype (h) and nucleotide diversity (π) and GENALEX to calculate population pairwise D_{est}.

Introgression estimates

To identify hybrid individuals, we used the microsatellite genotypes and a Bayesian clustering method implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000) to separate parental (MN or pre-translocation WI) and admixed ('hybrid') individuals. First, we estimated appropriate thresholds for separating these groups by simulating hybrid genotypes in HYBRIDLAB (Nielsen et al., 2006). We started with the observed microsatellite allele frequencies in the translocated MN female and pre-translocation WI samples, and, using HYBRIDLAB, we constructed equal sized samples (41) of the parental populations (translocated MN females, pre-translocation WI) and their F1, F2 and F1 backcross hybrids. These simulated individuals were analyzed with STRUCTURE using admixture models with prior population information (Figure S1.1). In this case, we used the two known sampling locations (pre-translocation WI and translocated MN females) to assist in ancestry estimation in our unknown sample (post-translocation WI, which was not assigned a population), as recommended when testing for hybrids (Pritchard et al., 2000; STRUCTURE manual pg 11). Finally, we assigned the post-translocation WI individuals to purebred or hybrid categories based on the 10 to 90th percentiles of population membership (Q) from the simulated hybrids. To visualize introgression in terms of allele frequencies, we

performed a Principal Coordinates Analysis (PCoA) in GENALEX (using the covariance matrix) and plotted the scores from the three sample groups on the first two axes.

Results

Microsatellites

By genotyping the translocated females, we were able to identify eight new microsatellite alleles that introgressed into WI. These alleles were found in the post-translocation sample (mean ± SD frequency: 0.012 ± 0.010), as well as the translocated MN sample (0.051 ± 0.047), but not in the pre-translocation sample. In total, there were 71 alleles found in the pre-translocation WI sample and of these, 60 (84%) were also found in WI after the translocation. Thus, 11 alleles were apparently lost. These 11 alleles were at lower frequency (mean ± SD: 0.02 ± 0.019) than the 60 alleles that were found in both the pre- and post-translocation samples (0.11 ± 0.140; unequal variances $t_{67.3}$ = 4.50, P < 0.0001), suggesting they were lost due to drift. In addition, four new alleles were found in the post-translocation WI sample that were not present in our sample of translocated females. These alleles were possibly the result of mutation or introduced by the translocated females that were not sampled (n = 24). Overall, there was a net gain of one allele (1.4% change) in the post-translocation WI population.

Over the seven generations from 1996 to 2011, we would have expected a significant loss (19%) of allelic richness at microsatellites (from 10.14 to 8.17; paired t_6= 3.19, P = 0.019) based on GENELOSS simulations of drift without translocation (Figure 1.2). However, there was no decline in allelic richness between the pre- and post-translocation samples (paired t_6 = 1.48, P = 0.188). We also found no difference in expected heterozygosity following the translocation (paired t_6 = 0.03, P = 0.971). There was also no difference between the post-translocation and

predicted (GENELOSS) estimate of allelic richness (paired $t_6 = 1.55$, $P = 0.22$; Figure 1.2).

Estimates of population structure (D_{est}) were significant between all pairs of populations (pre-

and post-translocation WI and translocated MN females) at microsatellites (Table 1.2).

mtDNA

There were originally ten mtDNA control region haplotypes from individuals sampled at the

Buena Vista marsh in the early 1950s. After a population bottleneck, the number declined to six

haplotypes (pre-translocation WI), but following the translocation of MN females the number

increased back to ten haplotypes (post-translocation WI). Thus, there was a net increase of four

haplotypes following the translocation (Table 4, Figure S1.2), which led to increases in both

haplotype (42%) and nucleotide (7%) diversity that approached historic levels in WI (Table 1.4,

Figure S1.2). As with microsatellites, there was significant population structure (θ_{ST}) between all

three sample groups at the mtDNA control region (Table 1.3).

MHC class I

No alleles were lost or gained between the pre- and post-translocation WI populations (Table

1.2). Three alleles were unique to the translocated MN females, but they occurred at a lower

frequency (0.036 - 0.073) than the alleles that were shared between MN and WI (0.036 - 0.255, n

= 6; $\chi^2 = 55$, df = 8, $P < 0.001$; Table S1.2, supporting information). There was no difference in

MHC class I haplotype diversity or the number of alleles between the pre- and post-translocation

samples in WI (Table 1.2). Allele frequencies also did not differ between pre- and post-

translocation WI ($\chi^2 = 5.68$, df = 7, $P = 0.58$); however, allele frequencies were different between

translocated MN and pre-translocation WI samples ($\chi^2 = 29.1$, df = 10, $P = 0.001$; Table S1.2).

Population structure (D_{est}) showed similar patterns with no difference between pre- and post-

translocation WI, but significant differentiation between the WI samples and the translocated MN sample (Table 1.3).

Introgression estimates

Among the post-translocation WI samples, 7% (6/81) were apparently admixed individuals (i.e., "hybrids") based on their microsatellite data and assignment using STRUCTURE. These individuals had probabilities of population membership for MN (mean ± SE: 0.55 ± 0.081; range 0.256 - 0.80) that were within the 10-90th percentile of values for simulated hybrids (0.235 to 0.881). There were also 16 individuals with unique MN alleles that could be considered admixed; 12 of these were not considered admixed by the STRUCTURE method because they had Q scores below the 0.253 threshold. Using both methods, the percentage of individuals with evidence of admixture from microsatellites was 22% (18/81). Evidence of introgression can also be seen in the substantial overlap between the post-translocation WI samples and the parental populations in a plot of microsatellite allele frequencies on the first two principal coordinates (Figure 1.3).

We can also use mtDNA haplotypes to identify admixed individuals. There were 24 individuals in the post-translocation sample (33%, 24/72) with MN haplotypes (haplotypes 7, 22, 48 and 139; Table 1.4) that were not found in the pre-translocation WI sample. Two of these haplotypes (7, 48) were previously found in the historic WI sample, and one (7) was fairly common (32%, 6/19; Table 1.4), suggesting that it was lost from the historic population and reintroduced by the translocated MN females. If we combine information from both the microsatellite and mtDNA markers, then the percentage of admixed individuals in the post-translocation sample was 47% (34/72 typed at both markers).

13

Introgression efficiency

We examined the efficiency of introgression in more detail by examining the genotypes of translocated MN females that were known to survive and breed. Following translocation from MN, 45 of 86 (52%) genotyped females were known to survive to their first breeding season in WI (based on radio-tracking). All females attempted to breed, but only 22 (26%) of these were known to have produced offspring based on hatched eggs at the nest. Collectively, these females explain seven of eight (88%) microsatellite alleles and both mtDNA haplotypes (haplotypes 22 and 139) that were successfully introduced into Wisconsin. However, these females also possessed 17 microsatellite alleles and 13 mtDNA haplotypes that were potentially new, but were not found in the post-translocation WI sample. At MHC class I, three novel alleles from MN were detected in the translocated MN females that successfully bred; however, these alleles were absent in the post-translocation WI sample.

Discussion

Translocations have been widely used to restore genetic variation, but few studies have examined their efficacy at both neutral and functional genes (Table 1.1). Our study found introgression of new alleles from MN into the isolated WI population, although it varied depending on the genetic marker. Even though the translocation did not increase genetic variation across all three marker types, it might be considered a success nonetheless, because our simulation model suggested that genetic drift would have caused a substantial loss of genetic variation in the absence of the translocation. In general, our results caution that detecting introgression varies according to the type of genetic marker and that successfully restoring genetic diversity may

require more individuals (or better reproductive success of the translocated individuals),

particularly in small, declining populations in which drift is strong.

Successful translocation depends on the ability of translocated individuals to contribute

their genes to future generations. Radio-tracking released individuals suggested that

introgression of new alleles was relatively low. For instance, the MN females that were known to

breed successfully had 17 microsatellite alleles, nine mtDNA haplotypes and three MHC class I

alleles that were potentially new, but were not found in the post-translocation WI sample. It is

possible that we missed these in the post-translocation sample because of sampling error, but

sample sizes were similar in the post-translocation WI and translocated MN samples (Table 1.2).

The level of admixture in the post-translocation sample reached 47% of individuals (using data

from all markers), and it might have been greater, but there was relatively low nesting success.

During this study 38% of translocated MN and 42% of native WI females hatched eggs from

their nest, which is below the 46-55% average of eight other studies in WI (Toepfer, 2003; Hull

et al., unpubl. data). Overall, we achieved our goal of restoring mtDNA diversity, but it may

require higher nesting success (or more translocated birds) to restore levels of microsatellite

diversity.

Successful introgression in small populations also depends on the opposing effects of

drift. In our case, simulations of drift predicted a continual loss of microsatellite diversity, yet no

change in allelic richness was observed between the pre- and post-translocation WI samples.

Drift continues to affect the WI population, as suggested by the loss of 11 microsatellite alleles

during the time period between pre- and post-translocation samples. However, introgression of

eight novel alleles from MN females apparently counteracted some of the eroding effects of drift

and helped to maintain microsatellite allelic diversity. Despite the introgression of microsatellite

alleles, there was no change in heterozygosity, which typically lags behind changes in the number of alleles (Allendorf, 1986). The current stability in genetic diversity is probably only temporary, and, if the WI population remains small, drift will quickly reduce genetic diversity gained through these supplemental translocations.

In contrast to microsatellites, we found an increase in mtDNA diversity following the translocations. Five haplotypes were apparently introduced by the translocation; however, there are alternative explanations for the presence of three haplotypes (7, 48, and 140) other than introgression. Haplotypes 7 and 48 were present in historic (1950s) WI and may be missing from the pre-translocation sample due to sampling error. While this may be the case for haplotype 48, the high frequency of haplotype 7 in both the historic WI (32%) and post-translocation (21%) samples suggests the translocation either re-introduced or restored its frequency to historic levels. Interestingly, haplotype 140 is common (10%) in the post-translocation WI sample, but it is absent in our translocated MN samples. While it is possible that haplotype 140 came from translocated individuals that were not sampled ($n = 24$), this haplotype is just one base pair different than haplotype 5 (Figure S1.2), which is also absent in our MN sample. Therefore, haplotype 140 could be the result of a single mutation that occurred within WI.

The extent of introgression will also vary with the type of inheritance of the genetic marker. The mitochondrial genome is haploid and is usually maternally inherited, whereas nuclear genes such as microsatellites are diploid and inherited from both parents. In the post-translocation WI samples, we detected proportionally more individuals with mtDNA haplotypes (33%, 24/72) than microsatellites alleles (22%, 18/81) that were unique to MN. Differences in introgression between mitochondrial and nuclear (e.g. microsatellites) DNA markers, particularly in studies of hybridization, are often explained as a consequence of adaptive introgression of

16

mtDNA or sex-biased dispersal (Karl et al., 2012; Toews and Brelsford, 2012). In our case, the difference may be due to translocating female birds, which had a large number of mtDNA haplotypes ($n = 20$) compared to the pre-translocation WI sample ($n = 6$; Table 1.4). Additionally, detecting introgression is more likely if one type of marker has more novel alleles, and in this study there were more novel mtDNA haplotypes (70%, 14/20) than novel microsatellite alleles (32%, 35/110) in the translocated MN samples (Table 1.1). Only three other studies have used both mtDNA and microsatellites to assess changes in genetic diversity following translocations to threatened populations (Table 1.1). Two of these studies showed increased genetic diversity at both markers following translocation (Bouzat et al., 2009; Olson et al., 2012), while the other study found an increase in mtDNA diversity, but no increase in microsatellite diversity (Arrendal et al., 2004), similar to our study.

Few genetic studies of supplemental translocation (e.g., Table 1.1) have been repeated on the same species, so it is worth comparing our results with a previous translocation of greater prairie-chickens into Illinois (IL) (Bouzat et al., 2009). In IL, it was estimated that only 50 individuals were left in the population by 1994 and both fertility and hatching rate of eggs had declined markedly (Westemeier et al., 1998). To prevent local extinction, managers translocated 518 birds to IL (Svedarsky et al., 2000), and five years following the last translocation (1998) haplotype diversity of mtDNA had increased by 20% to near historic (1930s) levels (Bouzat et al., 2009), similar to our results in WI (Table S1.3). When we compared the same four microsatellite loci used in both studies we found that expected heterozygosity increased in both populations (4% in WI and 2% in IL), but allelic richness increased 22% in IL (4.9 to 6.0) and decreased (non-significantly) 2.5% in WI (7.34 to 7.15; Table S1.3). This difference between IL and WI in changes to allelic richness might be explained at least partially by the larger numbers

of birds translocated into IL (518 birds were translocated to a population of about 50 birds), than in WI (110 birds were translocated into a population of about 550 birds). In nine previous genetic studies (Table 1.1), the median ratio of translocated individuals to total population size was 0.32 (range 0.06 to 10.4), and most studies showed an increase in genetic diversity after the translocation (Table 1.1).

Previous studies have focused on neutral genetic markers, which may not reflect adaptive variation (Väli et al., 2008). Since the function of MHC-derived proteins is to detect pathogens and activate the immune system, threatened populations with reduced MHC diversity may have increased susceptibility to disease (Siddle et al., 2007). In general, MHC variation appears to be reduced by drift in small populations (Eimes et al., 2011; Ejsmond and Radwan, 2011). As a result, there is an increasing awareness that measuring MHC variation will be important in assessing the results of supplemental translocations, but so far studies are rare. A study of European adders (*Vipera berus*) showed that MHC class I diversity increased following the translocation of 20 males into an isolated population of approximately 30 adults (Madsen et al., 1999). In contrast, we did not detect any changes in MHC class I diversity in prairie-chickens, probably in part because there were just three potentially novel alleles in the translocated females and they were at low frequency. However, in the absence of the translocation we would likely have seen a loss of MHC diversity due to drift (Eimes et al., 2011), as was predicted for microsatellite variation from our simulation model. Indeed, introgression and selection might have limited the effect of drift on MHC diversity. In particular, many of the females from MN had the same MHC alleles as WI birds, and, thus, introgression could have occurred and helped to maintain diversity, but remained undetected. Some studies suggest that selection can maintain MHC alleles in small, isolated populations (Aguilar et al., 2004; van Oosterhout et al., 2006).

This is also a possiblity in our study, but it will be difficult to determine because there were no MHC alleles unique to WI in our sample.

Most supplemental translocations appear to have successfully increased or maintained genetic diversity (Table 1.1). However, our study illustrates that interpreting these results may depend on the genetic markers used for evaluation. For instance, the translocation in WI increased mtDNA diversity and restored it to historic levels, but microsatellie diversity was only maintained at pre-translocation levels. In terms of particular genotypes, however, there was introgression of mtDNA haplotypes and microsatellite alleles from MN. In contrast, the translocation did not appear to change the diversity or composition of MHC variation in the WI population, suggesting that it may be more difficult to restore diversity at functional loci. Few genetic studies of translocations have examined different types of markers (Table 1.1), so these issues have rarely been considered previously. Most studies use neutral markers such as microsatellites or mtDNA as indices of genome-wide variation and inbreeding. As the number of markers increases in future studies, the distinctions between neutral and selected (functional) markers and their importance to the goals of restoration will become increasingly important. Our study also illustrates that small, isolated populations can continue to lose genetic variation during translocations. Unless population size also increases following a translocation, genetic drift is likely to continue, making it necessary to continue to monitor genetic diversity, and possibly conduct additional translocations. Thus, translocations may only be short-term solutions to temporarily reduce the risk of inbreeding and local extinction (Hedrick and Fredrickson, 2010). The ultimate goal should be to reverse the decline in numbers, and, in the case of prairie-chickens, future management may need to focus more on increasing reproduction and survival to increase population size.

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Figure 1.1. Map of the historic (light shading) and current (dark shading) range of greater prairie-chickens in the central US.

Figure 1.2. Change in allelic diversity (allelic richness, $n = 41$) at microsatellite loci from pre- to post- translocation WI in relation to the predicted change based on GENELOSS simulations (Predicted WI). P values are indicated for comparisons between pair of groups (see horizontal lines across the top) analyzed using paired t-tests (matching for locus; $n = 7$ loci in each group). Boxes indicate medians (center bar), 25 and 75th percentiles (bottom and top of boxes, respectively) and whiskers indicate maximum and minimum observations.
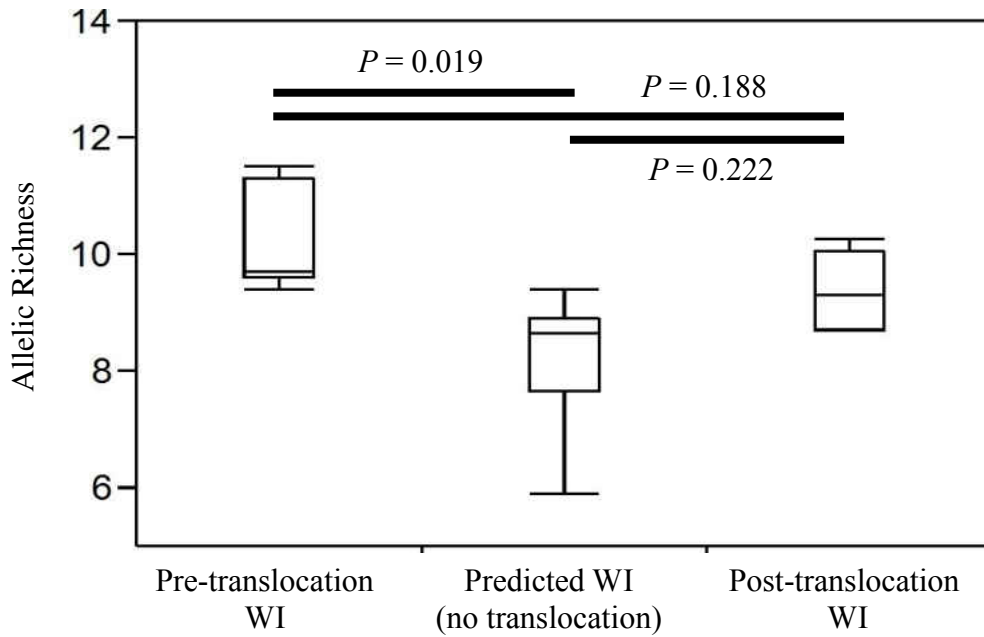
Figure 1.3. Microsatellite allele frequencies from a Principal Coordinates Analysis plotted on the first two axes. Frequency scores for translocated MN females are the solid black circles and dashed ellipsoid, pre-translocation WI = open circles and thin ellipsoid, post-translocation WI = open squares and thick ellipsoid. Ellipsoids contain 75% of observations.
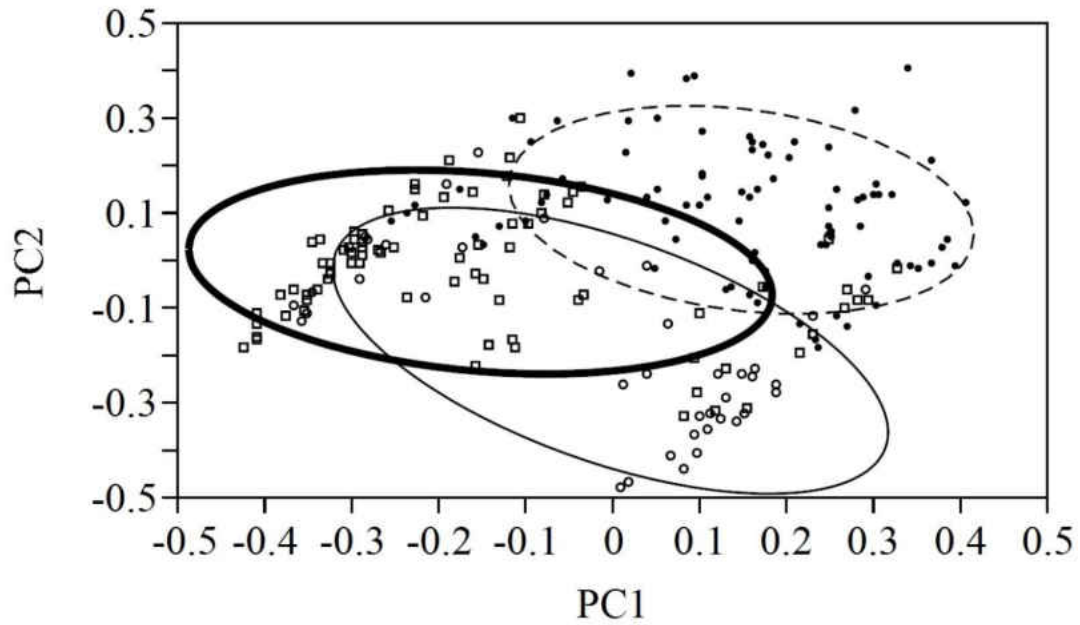
Figure S1.1. A) Population assignments of individuals from pre-, post-translocation WI and translocated MN females groups into three clusters (K = 3) using STRUCTURE with a model of admixture and no population information (based on microsatellite loci). The figure shows the membership coefficients (Q) for each individual (vertical line) to each cluster. B) Assignments of individuals into two clusters (K = 2) based on the admixture model with correlated frequencies with prior location information used for translocated MN females and pre-translocation WI only.

Figure S1.2. Median joining network displaying the relationship of mtDNA haplotypes in the three study groups. Each circle depicts a unique haplotype and the smallest circles correspond to sample sizes of one, and the size of larger circles is proportional to their haplotype frequency in the sample (see Table 1.4). Numbering of haplotypes follows Johnson et al. (2003, 2007). Color and pattern within a single haplotype circle correspond to groups where the haplotypes have been identified (solid gray = translocated MN females; solid black = post-translocation WI; crosshatch pattern = pre-translocation WI). Numbers on lines connecting haplotypes represent the exact position of nucleotide changes in the 384 bp mtDNA region examined. All novel haplotypes were submitted to GenBank (accession numbers: KF466480-KF466483).

Table 1.1. Comparison of supplemental translocation studies that included genetic analyses. Studies are based on human-facilitated translocations of free-ranging animals to threatened populations. Included are estimated population sizes before translocation (note that some studies examined two populations indicated by letters), number and sex of individuals translocated (n translocated), the number of individuals sampled pre- and post-translocation for genetic analyses, and whether genetic samples were analyzed of the translocated individuals (yes/no). For studies that used microsatellites (Msats), allelic richness (A_r) and expected heterozygosity (H_e) are presented.

Species	Population size pre-translocation	n translocated (sex)	Genetic sampling		Markers	Conclusions	Reference
			n sampled Pre, Post	Translocated individuals			
Greater prairie-chicken (*Tympanuchus cupido pinnatus*)	550	110 (F)	41, 81	yes	Msats, mtDNA, MHC	Significant increase of mtDNA diversity but no change of Msats and MHC diversity in post-translocation population.	This study
White-spotted charr † (*Salvelinus leucomaenis*)	?	K: 20 (F) H: 20 (F)	50, 48 52, 46	yes	Msats	Increased H_e and A_r diversity of post-translocation populations to levels of the source populations.	(Yamamoto et al., 2006)
California bighorn sheep‡ (*Ovis canadensis californiana*)	S: 185 L: 125	S: 15 (F) L: 16 (F)	19, 48 23, 50	yes	Msats, mtDNA	Increased mtDNA diversity (S & L) and significant increase of Msats H_e (L only) and A_r (S and L) in post-translocation populations following one generation.	(Whittaker et al., 2004; Olson et al., 2012)

Bighorn sheep (*Ovis canadensis*)	42	15 (8M, 7F)	20*, 115	no?	Msats	Increased H_e A_r and fitness in post-translocation population.	(Hogg et al., 2006)
Florida panther (*Puma concolor coryi*)	25	8 (F)	62, 67	yes	Msats	Increased H_e and A_r in post-translocation population. Decreased frequency of kinked tails and undescended testicles.	(Johnson et al., 2010, Table S3A)
Greater prairie-chicken	50	518 (M, F)	32, 18	no	Msats, mtDNA	Increased mtDNA diversity and increased Msat H_e and A_r in post-translocation population.	(Bouzat et al., 2009)
European adder (*Vipera berus*)	30	20 (M)	7, 7	no	MHC	Increased MHC diversity, number of recruiting males and population size.	(Madsen et al., 1999)
South Island robin§ (*Petroica australis*)	A: 60 M: 300	A: 13 (F) M: 18 (F)	45, 50 82, 122	no	Msats	Significant increase of A_R and H_e in post-translocation populations. Increased juvenile survival and recruitment, sperm quality and immune response.	(Heber et al., 2013)
Anacapa deer mouse (*Peromyscus maniculatus anacapae*)	173	1023 (M,F)	185, 60	no	Msats	Significant increases of H_e and A_R two years after translocations.	(Ozer et al., 2011)

| Eurasian otter (*Lutra lutra*) | ? | 54 (M, F) | 15, 20 | no | Msats, mtDNA | Increase of mtDNA diversity. Decrease in Msat diversity. | (Arrendal et al., 2004) |

† Studied the effects of translocations upstream of dams in two populations: Kame River (K) and Hitozuminai River (H), Hokkaido Island, Japan

‡ Experimental translocations into two populations: Steens Mountain (S) and Leslie Gulch (L), Oregon, USA

* sampled 20 descendants of original population in 1985 before translocations

§ Reciprocal translocations between two populations: Allports Island (A) and Motuara Island (M), Marlborough Sounds, New Zealand

Table 1.2. Genetic diversity at seven microsatellite loci, mtDNA control region and MHC class I in three prairie-chicken samples (years sampled): pre-translocation WI (1996-1999), post-translocation WI (2011) and translocated MN females (2006-2009). Differences between means (±SE) of pre-translocation and post-translocation WI were tested with t-tests controlling for locus. Alleles or haplotypes were considered private if absent from the other sample groups.

Population	Pre-translocation WI	Post-translocation WI	Percent Change	t, P (df)	Translocated MN females
Microsatellites					
N	41	81			86
Number of alleles	71	72	1.4		110
Private alleles	1	4			35
Allelic richness	10.14 (0.488)	9.11 (0.584)	−10.2	1.48, 0.188 (6)	13.38 (0.818)
H_o	0.683 (0.046)	0.716 (0.017)	4.8	0.80, 0.452 (6)	0.684 (0.058)
H_e	0.726 (0.025)	0.725 (0.023)	−0.1	0.03, 0.971 (6)	0.764 (0.047)
mtDNA					
N	41	72			86
Haplotypes	6	10	66.6		20
Private haplotypes	0	1			14
Haplotype diversity (h)	0.577 (0.082)	0.822 (0.023)	42.5	0.004[†]	0.920 (0.014)
Nucleotide diversity (π)	0.013 (0.001)	0.014 (<0.001)	7.7		0.009 (<0.001)
MHC class I					
N	30	31			26
Number of alleles	8	8	0		9
Private alleles	0	0			3
Haplotype diversity (h)	0.837 (0.022)	0.812 (0.022)	−3.0	0.329[†]	0.844 (0.025)
Nucleotide diversity (π)	0.034 (0.003)	0.031 (0.002)	−8.8		0.041 (0.002)

* P values comparing pre- and post-translocation WI haplotype diversity using the double-testing procedure of (Weale, 2003); algorithm TEST_h_DIFF, available at http://www.ucl.ac.uk/tcga/software/index.html

Table 1.3. Fixation indices for comparisons from seven microsatellite loci (D_{est}), control region of mtDNA (θ_{st}) and MHC class I (D_{est}) between greater prairie-chicken groups from Wisconsin (pre- and post-translocation WI) and translocated MN females.

Marker	Pre-WI vs. Post-WI	Pre-WI vs. Trans MN	Post-WI vs. Trans MN
Msats	0.116**	0.243**	0.236**
mtDNA	0.097**	0.227**	0.084**
MHC class I	0.009	0.218*	0.130*

*$P < 0.05$; ** $P < 0.001$

Table 1.4. Number of mtDNA control region haplotypes in greater prairie-chickens from historic (1952-1954), pre- and post-translocation WI at Buena Vista Wildlife Management Area, and the translocated MN females. Data from 1952-1954 are from Table S2 in Johnson et al. (2007). Thirteen haplotypes found only in the translocated MN females were combined in a single row (MN private). Sample sizes are shown in parentheses.

Haplotype ID	Historic WI (*n* = 19)	Pre-trans WI (*n* = 41)	Post-trans WI (*n* = 72)	Trans MN females (*n* = 86)
1	2	26	21	
2	2	6	15	
3		2	2	
4	1	3	1	11
5		3	2	
7	6		15	17
12	1			
15	1			
21	1	1		5
22			4	6
41	2			
42	1			
43	2			5
48	3[†]		1	2
139			4	2
140			7	
MN private				38
N haplotypes	11	6	10	20

[†] Found historically in the Mead Wildlife Management Area (WMA), which is now genetically distinct from the Buena Vista WMA (Johnson et al., 2007).

Table S1.1. Number of alleles (N_a) and observed (H_o) and expected (H_e) heterozygosity at microsatellite loci in the three prairie-chicken samples (pre-translocation WI; translocated MN females; post-translocation WI). Samples sizes were 41, 86 and 81, respectively.

	N_a			H_o			H_e		
	Pre-WI	Trans MN	Post-WI	Pre-WI	Trans MN	Post-WI	Pre-WI	Trans MN	Post-WI
ADL146	6	8	6	**0.146**	**0.523**	**0.272**	0.560	0.782	0.564
ADL230	15	21	16	0.756	**0.872**	**0.877**	0.816	0.915	0.884
LLSD4	12	21	14	0.854	0.895	**0.975**	0.846	0.918	0.858
LLST1	5	7	5	0.268	0.512	0.383	0.337	0.518	0.351
SGCA11	7	9	7	0.829	0.430	0.765	0.759	0.399	0.748
SGCA6	13	26	15	0.927	**0.663**	**0.827**	0.895	0.907	0.866
SGCA9	13	18	9	1.000	0.895	**0.914**	0.870	0.915	0.808

Bold H_o values indicate deviations from HWE.

Table S1.2. Nucleotide alignment of the 11 observed MHC class I exon 3 sequences (373 - 379 bp) found in the three groups of greater prairie-chickens (pre-, post-translocation WI and translocated MN females). Vertical numbers indicate the position of the 33 variable nucleotides sites within exon 3. Sites marked with dots indicate the same nucleotide as Tycu-1A*15 allele and dashes represent deletions. The MHC class I allele frequencies and the allele counts (*n*) are provided for each group. Novel MHC class I sequences were submitted to GenBank (accession numbers: KF466475-KF466479).

```
                    1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2 2
         1 1 1 6 6 7 9 9 0 2 2 2 3 7 7 7 7 8 8 8 8 8 8 8 8 9 9 9 9 9 0 2 3
         2 3 4 7 8 3 3 9 5 2 3 4 5 6 7 9 0 1 2 3 5 6 7 8 9 1 5 6 8 9 5 9 9
```

	Sequence	Pre-trans WI (*n* = 62)	Post-trans WI (*n* = 66)	Trans MN (*n* = 55)
Tycu-1A*15	`G G T T A T T T G C G A G A T A T G G G G C G T T C G A G T C G A`	0.129	0.288	0.255
Tycu-1A*06	`A C G C G G C C C – – – . C A . . C C A C T T . . T T G A C . . .`	0.097	0.061	0.255
Tycu-1A*08	`A C G C G G C . . – – – . G . . G . . . . T . A A . . . . . . C G`	0.226	0.182	0.091
Tycu-1A*09	`. . . C G G C C C – – – . C A . . C C A C T T . . T T G A C . . .`	0.048	0.030	0.000
Tycu-1A*11	`. . . C G G C . . – – – . . . . . . . . . . . . . . . . . . . . .`	0.065	0.046	0.036
Tycu-1A*13	`T . . C G G C C C – – – . . . . . . . . . . . . . . . . . . . C G`	0.129	0.136	0.127
Tycu-1A*14	`T . . C . G C C C – – – . . . . . . . . . . . . . . . . . . . C G`	0.274	0.242	0.091
Tycu-1A*16	`T . . C G G C . . – – – . G . . G . . . . T . A A . . . . . . C G`	0.000	0.000	0.036
Tycu-1A*17	`. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G`	0.000	0.000	0.036
Tycu-1A*18	`. . . C . G C C C – – – A . A – – – T . . T . A A . . . . . A . G`	0.000	0.000	0.073
Tycu-1A*19	`T . . C . G C C C – – – . . . . . . . . . . . . . . . . . . . . G`	0.032	0.015	0.000

Table S1.3. Mean (SE) estimates of mitochondrial (mtDNA) and microsatellite (Msat) DNA diversity in greater prairie-chickens for pre- and post-translocation in Wisconsin (Buena Vista marsh, this study) and Illinois (Bouzat et al., 2009). For microsatellites, statistics were calculated for only the four loci used in both studies (ADL146, ADL230, LLSD4, LLST1).

	Wisconsin			Illinois		
	Historic*	Pre-translocation	Post-translocation	Historic	Pre-translocation	Post-translocation
mtDNA						
Sample size	19	41	72	22	32	18
Number of haplotypes	10	6	10	8	4	7
Haplotype diversity (h)	0.889 (0.013)	0.577 (0.082)	0.822 (0.023)	0.883 (0.007)	0.728 (0.007)	0.876 (0.009)
Nucleotide diversity (π)	0.012 (0.001)	0.013 (0.001)	0.014 (<0.001)	0.007 (0.000)	0.005 (0.000)	0.013 (0.000)
Msats						
Sample size	40-45	41	81	–	32	18
Allelic richness (A_r)	8.2 (1.300)	7.34 (0.404)	7.15 (0.721)	–	4.94 (0.451)	6.00 (0.836)
H_o	0.653 (0.122)	0.506 (0.175)	0.627 (0.175)	–	0.625 (0.082)	0.639(0.117)
H_e	0.696 (0.099)	0.640 (0.120)	0.664 (0.127)	–	0.656 (0.084)	0.667 (0.079)

* from Bellinger et al. (2003), Johnson et al. (2004)

Supplemental Molecular Methods

Sexing prairie-chicken feathers. The sex of individuals sampled from post-translocation WI was determined by PCR using primers that amplify an intron of the CHD gene located on the W and Z sex chromosomes (Kahn et al., 1998). The intron varies in size between the two chromosomes resulting in two bands for females (ZW) and one band for males (ZZ). PCR was carried out in a total volume of 15 μL with the following conditions: 1X GoTaq Flexi Buffer (Promega), 1.65 mM MgCl$_2$, 0.8 mM dNTPs, 1.0 μM of each primer, 0.5 U of GoTaq polymerase, 2% DMSO and approximately 50 ng of genomic DNA. PCR amplifications were performed under the following thermocycling conditions: an initial denaturing step at 94 °C for 2 min. followed by 30 cycles at 94 °C for 30 s, 56 °C for 45 s and 72 °C for 45 s, and a final extension at 72 °C for 5 min. PCR products were visualized on a 3% NuSieve 3:1 agarose (FMC Corp.) gel, run at 23 V/hr and stained with ethidium bromide. DNA of known sex individuals (2 males and 2 females) was amplified in each PCR and run on each gel as positive controls. We conducted three separate PCRs of each sample to avoid false typing of individuals that may occur with DNA extracted from feathers (Bush et al., 2005). Sequencing the PCR products from one male and one female revealed the length of the intron amplified on the Z chromosome was 224 bp and the W chromosome was 256 bp.

MHC class I cloning and sequencing. We examined MHC diversity at exon 3 of the class I loci. Based on our genetic map of the core MHC-B in greater prairie-chickens (Eimes et al., 2013), we developed two new primers 1a2inExon3F (5′-TCACCTCTCCTGCCCAGCTC-3′) and 1a2Raltintron2 (5′- ATCCCCTGCCCGGCTGTGTT-3′) that amplify exon 3 of MHC class I loci. PCRs (20 μL total volume) contained 1x Green GoTaq Flexi Buffer (Promega), 1.5 mM of

MgCl$_2$, 7.5% DMSO, 0.5 µM of each primer, 0.4mM of dNTPs, 1.0 unit of GoTaq DNA polymerase and approximately 50 ng of genomic DNA. Thermocycling conditions started with an initial denaturation step at 95 °C for 1 min. followed by 30 cycles of 30 s at 94 °C, 30 s at 66 °C and 45 s at 72 °C. We confirmed amplifications on 1% agarose gel. Initially, PCR products were sequenced, but 89% (77/87) of the generated chromatograms were difficult to interpret due to indels resulting in allelic length heterogeneity for each individual. Therefore, we purified PCR products using GeneJet Gel Extraction Kit (Fermentas) and cloned using pGEM®-T Easy Vector System II (Promega, Madison, WI) with JM109 competent cells. For whole colony PCR, the colonies to be screened were sampled with a sterile 10-µl pipette tip and transferred to a PCR mixture containing 1x Green GoTaq Flexi Buffer, 1.5 mM of MgCl$_2$, 0.5 µM of each vector primer (Sp6 and T7), 0.4 mM of dNTPs and 1.0 unit of GoTaq DNA polymerase. Thermocycling conditions consisted of an initial denaturation for 2 min. at 94 °C followed by 30 cycles of 15 s at 94 °C, 15 s at 40 °C, 45 s at 72 °C with a final extension for 5 min. at 72°C. We identified alleles in two ways: 1) cloning of 77 individuals (6 colonies per individual) and 2) direct sequencing of 10 individuals in both directions and aligned with the cloned alleles in the program PHASE (Stephens et al., 2001) implemented in DNASP. All sequences were aligned with MHC class I exon 3 sequence of the domestic chicken (*Gallus gallus*; Genbank No. AB426152.1) using the program GENEIOUS.

Introgression estimates. We used two methods to estimate the introgression of MN alleles into the WI population. First, we calculated a hybrid index as described in the main text. Our second method to estimate introgression at microsatellites used a Bayesian Monte Carlo Markov Chain (MCMC) algorithm in STRUCTURE 2.3.4 (Pritchard et al., 2000) to assign individuals into genetic

clusters (K) and estimate their probability of membership in each cluster (Q). All individuals

were tested with independent simulations of K, ranging from 1 to 5, with each level of K

replicated 10 times. Each simulation was run for 500 000 MCMC steps after a burnin of 50 000.

We used STRUCTURE HARVESTER 0.6.93 (Earl and vonHoldt, 2012) to combine the results from

the 10 independent simulations for each value of K. The number of clusters (K) was determined

based on the ΔK method (Evanno et al., 2005).

To identify hybrid individuals, we simulated hybrids and used STRUCTURE to estimate

appropriate thresholds of population membership (Q values) for separating parental (MN or pre-

translocation WI) and admixed ('hybrid') individuals (K = 2 in all analyses). Starting with the

observed allele frequencies in the translocated MN female and pre-translocation WI samples, we

used HYBRIDLAB (Nielsen et al., 2006) to construct equal sized samples (41) of the parental

populations (translocated MN females, pre-translocation WI) and their F1, F2 and F1 backcross

hybrids. These simulated individuals were analyzed with STRUCTURE using admixture models

with prior population information. In this case, we used the two known sampling locations (pre-

translocation WI and translocated MN females) to assist the ancestry estimation in our unknown

sample (post-translocation WI, which was not assigned a population), as recommended when

testing for hybrids (Pritchard et al., 2000; STRUCTURE manual pg 11). From the analysis of

simulated hybrids we used the 10 to 90th percentiles of population membership (Q) as a threshold

for assigning the post-translocation WI individuals to purebred or hybrid categories.

Using all three samples (pre-, post-translocation WI and translocated MN females), STRUCTURE

found the greatest support for three genetic clusters (K = 3; Figure S1.1A) in an admixture model

without assignment to populations. However, to test specifically for hybrids in the post-

translocation sample, we assumed two parental populations (pre-translocation WI and translocated MN females; K=2) and used the test for migrants model in STRUCTURE (Figure S1.1B). Membership probabilities (Q) for the post-translocation samples in the parental MN cluster ranged from 0.007 to 0.80 (median = 0.02, 10 to 90th percentiles: 0.011 to 0.16). Next, we used the 10 to 90th percentiles of simulated 'hybrids' (from HYBRIDLAB) to create thresholds (10 to 90th percentile) for identifying individuals with mixed ancestry in our post-translocation WI samples. These Q values were 0.235 and 0.811, respectively. Based on these thresholds, 7% (6/81) of birds had MN ancestry (mean ± SE for Q: 0.55 ± 0.081; range 0.256 - 0.80) in the post-translocation WI sample (see main text for more details).

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Contrasting patterns of selection and drift between two categories of immune genes in prairie-chickens

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Abstract

Immune-receptor genes of the adaptive immune system, such as the major histocompatibility complex (MHC), are involved in recognizing specific pathogens and are known to have high rates of adaptive evolution, presumably as a consequence of rapid coevolution between hosts and pathogens. In contrast, many 'mediating' genes of the immune system do not interact directly with specific pathogens and are involved in signaling (e.g., cytokines) or controlling immune cell growth. As a consequence, we might expect stronger selection at immune-receptor than mediating genes, but these two types of genes have not been compared directly in wild populations. Here, we tested the hypothesis that selection differs between MHC (class I and II) and mediating genes by comparing levels of population differentiation across the range of greater prairie-chickens (*Tympanuchus cupido*). As predicted, there was stronger population differentiation and isolation-by-distance at immune-receptor (MHC) than at either mediating genes or neutral microsatellites, suggesting a stronger role of local adaptation at the MHC. In contrast, mediating genes displayed weaker differentiation between populations than neutral microsatellites, consistent with selection favoring similar alleles across populations for mediating genes. In addition to selection, drift also had a stronger effect on immune-receptor (MHC) than mediating genes as indicated by the stronger decline of MHC variation in relation to population size. This is the first study in the wild to show that the effects of selection and drift on immune genes vary across populations depending on their functional role.

43

Introduction

Individuals are continually challenged by a wide variety of pathogens, which has resulted in the evolution of a diverse and complex immune system. Immune genes are categorized based on their immunological roles, each of which experiences different selection pressures (Downing *et al.* 2010; Ekblom *et al.* 2010). In particular, genes of the adaptive immune system, such as the major histocompatibility complex (MHC), are involved in recognition of specific antigens and experience strong selection as a consequence of host-pathogen coevolution ("immune receptor" genes). In contrast, "mediating genes" are involved in signaling (e.g., cytokines) or controlling immune cell growth (e.g., inhibitor of apoptosis protein-1, IAP-1) and are thought to experience weaker selection because they have more general roles that do not involve recognition of specific pathogens (Sackton *et al.* 2007; Ekblom *et al.* 2010). With the rapid increase of novel pathogens that may threaten local populations with extinction (Smith *et al.* 2009), it is important to understand how a broad range of immune genes respond to contemporary selection in the wild.

Many studies investigating selection at immune-receptor genes have focused on the MHC, which initiates the adaptive immune response by recognizing foreign antigens produced by pathogens and presenting them to T cells (Janeway *et al.* 2005). Coevolution between pathogens and immune system is considered the primary force driving MHC variation (Bernatchez & Landry 2003). MHC allele frequencies may be similar among populations if beneficial alleles quickly increase in frequency (Schierup *et al.* 2000; Muirhead 2001), assuming that pathogen-driven selection is similar among populations and selection prevents the loss of rare beneficial alleles due to drift. On the other hand, MHC alleles that are beneficial in one population may not be as beneficial in other populations with a different array of pathogens. In this case, locally adapted MHC alleles may exist leading to greater population differentiation (Muirhead 2001). To

date, studies have found that, compared to neutral markers, MHC differentiation can be: (1) greater than expected suggesting local adaptation (Ekblom *et al.* 2007; Loiseau *et al.* 2009; Ackerman *et al.* 2013; Kyle *et al.* 2014), (2) weaker than expected consistent with balancing selection (Sommer 2003; Fraser *et al.* 2010; Strand *et al.* 2012), or (3) not different than neutral markers suggesting weak selection or a strong effect of drift (Miller *et al.* 2010; Zeisset & Beebee 2014). These differences in results may be related to the relative effects of gene flow, drift and selection (Bryja *et al.* 2007) and the spatial scale of the study (Landry & Bernatchez 2001, Herdegen *et al.* 2014). For example, at small spatial scales MHC differentiation could be greater than that of neutral genes because of local adaptation, but at larger spatial scales MHC differentiation could be reduced because gene flow of adaptive MHC alleles is likely to be greater than that of neutral genes (Herdegen *et al.* 2014).

In contrast, few studies have explored population differentiation at other immune genes. Most of these studies focus on toll-like receptors (TLRs; Ryan *et al.* 2006), another type of immune-receptor gene, or meditating genes such as cytokines, which appear to have relatively low variation and population differentiation compared to neutral markers (Bollmer *et al.* 2011). Many mediating genes are responsible for signaling and controlling immune responses (e.g., inflammation and immune cell growth) so they do not interact directly with pathogens. As a consequence, selection for genetic variability or local adaptation in these genes may be weaker (Sackton *et al.* 2007), potentially leading to similar alleles across populations. Here we tested the hypothesis that selection differs between immune-receptor (MHC) and mediating genes by comparing levels of population differentiation at these genes in natural populations of greater prairie-chickens (*Tympanuchus cupido*). Although many studies have examined selection and drift in MHC genes, they have not been compared directly to mediating genes in analyses of wild

populations. There is also mounting evidence that variation declines faster at MHC than neutral genes following bottlenecks (Esjmond & Radwan 2009, Eimes *et al.* 2011, Sutton *et al.* 2011), but it is not clear whether the decline is similar in other types of immune genes.

To examine the effects of selection and neutral processes, we compared immune genes in relation to neutral markers (microsatellites) across six populations of greater prairie-chickens that vary greatly in size (176 to >170000 birds) and, hence, the influence of drift. The populations are distributed over a large geographic area (up to 2000 km apart, Table S2.1) across 16° of latitude (30° - 46° N), which may expose them to different pathogen communities (e.g., Guernier *et al.* 2004). We previously examined variation within these prairie-chicken populations at five mediating genes (see Methods) and found a positive relationship between population size and genetic variation, consistent with the effects of drift (Bollmer *et al.* 2011). However, population differentiation was weaker at these mediating genes than neutral markers, suggesting that selection has favored similar alleles across populations (Bollmer *et al.* 2011).

In our present study, we compare patterns of geographic variation at two immune receptor genes (MHC class I and II) with the five mediating genes described in Bollmer *et al.* (2011). Few studies examine both MHC class I and class II loci, which code for antigen receptors that detect intracellular and extracellular pathogens, respectively. We predicted that if host-pathogen selection maintains similar MHC alleles across populations, then we would expect weaker levels of differentiation at MHC than neutral genes, similar to the mediating genes. On the other hand, if there were local adaptation at MHC genes, then we would expect stronger population differentiation at MHC genes than at both neutral markers and mediating genes.

Methods

We sampled 183 individuals from six populations of greater prairie-chickens (*T. c. pinnatus*) in Kansas, Nebraska, Minnesota, Missouri, Wisconsin (*n* = 30 each) and the endangered Attwater's subspecies in Texas (*n* = 33; *T. c. attwateri*). We sampled birds from one or two adjacent counties in each state (see map in Johnson *et al.* 2003) to limit the potential effects that sampling from areas of unequal size may have on measures of genetic diversity. DNA was extracted from blood and feathers collected during 1997-2011 following the methods described in Johnson *et al.* (2003). Most of the same individuals analyzed in Bollmer *et al.* (2011) at microsatellites and mediating genes were examined at MHC class I and II in this study, with the exception of samples from Minnesota and Texas. In these populations, additional individuals from different years were used to examine MHC variation. The MHC samples from Minnesota, consisted of individuals that were captured in 2006 and used in a supplemental translocation to the threatened Wisconsin population (Bateson *et al.* 2014). Note that the Wisconsin samples (from 1999-2000) were collected prior to the introduction of these Minnesota individuals. The additional Texas samples used for MHC analysis were collected in 2011 from the descendants of a captive breeding program founded by 19 lineages in the early 1990s (Morrow *et al.* 2004). Using a DNA-based pedigree, we avoided using closely related individuals from the Texas population that had pairwise relatedness coefficients greater than 0.125.

454 pyrosequencing of MHC class I and II

We assessed genetic variation at exon 3 of MHC class I and exon 2 of MHC class II loci. To amplify MHC class I, we used primers and PCR protocols described in Bateson *et al.* (2014). Once the flanking intron nucleotides were trimmed, the most common class I sequence length

47

was 258 bp. However, as found in other studies of MHC class I (Čížková *et al.* 2012; Sepil *et al.* 2012), there were insertions (position 40) and deletions (position 58) that resulted in 261 and 255 bp sequences, respectively. For MHC class II, we used primers Blex2F (Eimes *et al.* 2010) and RNA R1a (Strand *et al.* 2007) that amplified the entire 237 bp fragment of exon 2 (PCR conditions are described in Eimes *et al.* 2010). Greater prairie-chickens have a single class I locus and two copies (paralogs) of the class II locus (Eimes *et al.* 2013, Bateson *et al.* 2014); therefore, we amplified a maximum of two and four alleles within individuals, respectively. Due to the complexity of MHC class I (length polymorphism) and class II (multiple loci), we used 454 pyrosequencing to genotype individuals. Amplifications of MHC loci included fusion primers comprised of Roche 454 adapter sequences, an 8-bp barcode (to identify individuals) and a pair of either the MHC class I or II primers. Amplicons for both class I and II were pooled and then sequenced on a Roche 454 FLX Genome Sequencer using Titanium chemistry at Research and Testing Laboratory, LLC (Lubbock, TX).

Filtering MHC pyrosequences and allele validation

It is well known that 454 pyrosequencing is prone to errors (e.g., Gilles *et al.* 2011), which can artificially inflate the number of alleles within individuals and populations. Thus, several step-wise methods have been proposed to identify true alleles (e.g., Lighten *et al.* 2014a; Sommer *et al.* 2013). We processed and filtered our 454 sequences according to the methods of Lighten *et al.* (2014a, 2014b). First, we used jMHC v. 1.5 (Stuglik *et al.* 2011) to exclude 454 sequences that contained ambiguities (N's) and did not contain the entire forward primer, the correct 8-bp barcode (to identify individuals) and at least 10 bases of the reverse primer. Next, we excluded sequences that: 1) were found as a single read within amplicons (samples), or 2) differed by

more than 2 bp from the expected exon size because of PCR or sequencing errors. To identify

true alleles, we clustered the remaining sequences by building neighboring-joining trees in

Geneious version 7.0 (http://www.geneious.com, Kearse *et al.* 2012). Each cluster consisted of

one sequence with a high number of reads (the true allele) and error sequences which were

always $\leq$5% of reads in a cluster (similar to Lighten *et al.* 2014a). Each true allele was found in

at least two individuals (87%, 48/55) or verified in two independent PCRs and 454 sequencing

runs (13%, 7/55 alleles, see Repeatability of MHC genotyping below).

After applying the allele validation steps above, we estimated the number of MHC alleles

in each individual using two independent methods (copy number variation, CNV and degree of

change, DOC) described by Lighten *et al.* (2014a,b). Both methods estimate the number of

alleles from the 10 most common sequences within each amplicon, and, therefore, can estimate

the number of alleles for up to a maximum of five MHC loci.

The CNV method identifies the number of alleles and loci by using the proportion of

reads for each allele. If an allele has a relatively higher read count than others, then the allele

may exist at multiple loci within an individual. The observed read counts for each allele within

individuals are compared to a theoretical genetic model that calculates expected read counts for a

range of loci (1-5 loci) and allelic copies. Next we compared the estimated number of alleles

from the top two CNV models (with the lowest sum of squares). If the estimated number of

alleles differed between these two models, then we compared the lowest number of alleles for

that individual to the results of the DOC method.

The DOC method determines the number of alleles by identifying a substantial drop or

inflection point in the number of reads or the degree of change (DOC) that is expected between

alleles and artifact sequences. The DOC approach assumes that alleles have a higher number of

reads than artifact sequences, so there is a breakpoint in the number of reads between the least amplified allele and the most common artifact within each amplicon. The quality of an amplicon is important for the DOC method to reliably determine the number of alleles; therefore, we independently examined the cumulative number of reads for the 10 most common sequences for each amplicon to make sure there was an obvious breakpoint between the true alleles and artifact sequences.

The final MHC class I and II genotypes for each individual were determined when both the CNV and DOC methods converged on the same number of alleles. If the CNV and DOC methods disagreed, we used the DOC estimate, which is more conservative (Lighten *et al.* 2014b). There was agreement between the CNV and DOC approaches for 97% (178/183) of class I and 90% (164/182) of class II genotypes.

After our initial filtering steps, we obtained 153,241 reads for the 183 individuals at MHC class I, and 250,245 reads for 182 individuals at MHC class II. One individual from Minnesota failed to amplify at class II in two pyrosequencing runs. The average number of reads per individual was 701 for class I (range: 25–3124) and 1091 for class II (range: 145–6197). There was no correlation between total number of reads and alleles at class I ($r^2 = 0.01$, $F_{1,181} = 2.46$, $P = 0.118$) or class II ($r^2 = 0.01$, $F_{1,182} = 1.72$, $P = 0.191$), indicating sufficient sequencing coverage for genotyping. Overall, pyrosequencing detected 15 class I and 14 class II alleles in addition to those that were previously identified in Wisconsin and Minnesota prairie-chickens using cloning and Sanger sequencing methods (Eimes *et al.* 2010, Bateson *et al.* 2014).

Repeatability of MHC genotyping

We also examined the repeatability of our genotyping procedure at class I and II by comparing two independent PCRs and 454 sequencing runs for 18 individuals. These repeated samples were selected to confirm putative class I and II alleles found in a single individual and the number of class II alleles (range: 1-4) within individuals. Among the 18 individuals there were only three mismatches in the number of alleles per individual between runs, which resulted in high repeatability across both class I and II (R=0.93; $F_{35, 36}$ = 29.13; P < 0.001). The three mismatches all occurred at class I and appeared to be due to a low number of reads for those individuals in one 454 run. For 41 individuals we also compared MHC class I genotypes derived from pyrosequencing to those previously detected with cloning and Sanger sequencing (Bateson *et al.* 2014). Most (85%, 35/41) of the MHC genotypes matched. The six class I genotype mismatches were due to one allele missing in the cloned genotypes, the result of sequencing a small number of bacterial colonies (6 clones/individual).

MHC diversity

We used different measures to compare population genetic variation at class I and II because they have different numbers of loci. Class I has a single locus, so for population comparisons we calculated heterozygosity and tested for deviations from Hardy-Weinberg equilibrium using ARLEQUIN ver. 3.5 (Excoffier & Lischer 2010). Class II has two loci, and our primers amplify both of them simultaneously, so to compare variation at class II we calculated an index of allelic richness, theta k, using ARLEQUIN and the mean number of MHC alleles per individual (MHC/ind). Population-wide nucleotide diversity was calculated for both class I and II using DNASP v5 (Librado & Rozas 2009).

Mediating immune genes

We compared variation in MHC genes to the five mediating genes examined previously in the same populations as described in Bollmer *et al.* (2011). We focused on mediating genes that have polymorphisms correlated with health (e.g., pathogen load) and fitness-related traits in domestic chickens (Ye *et al.* 2006; Tohidi *et al.* 2013). The mediating genes examined were chicken B cell marker 6 (ChB6 or Bu-1), inhibitor of apoptosis protein-1 (IAP-1), interleukin-2 (IL-2), transforming growth factor β3 (TGF-β3) and tumor necrosis factor-related apoptosis inducing ligand-like protein (TRAIL-like). Briefly, ChB6 is a B lymphocyte receptor that induces cell death to self-reactive B cells, preventing autoimmune disease (Funk *et al.* 2003). The cytokine, IL-2 is involved in the activation of various immunity cells, including T cells and natural killer cells (Borish & Steinke 2003). IAP-1 prevents cell death and may be important in fighting intracellular pathogens (Prakash *et al.* 2009). TGF-β3 gene is a part of the cytokine family and has multiple immunological functions, including inhibiting the proliferation of lymphocytes, inducing immune cell migration and also suppressing inflammation (Borish & Steinke 2003). The cytokine, TRAIL-like protein stimulates apoptotic cell death, and activates cytotoxic T cells and IL-2-stimulated natural killer cells (Borish & Steinke 2003).

Microsatellite loci

We examined six microsatellite loci (methods in Johnson *et al.* 2003) to provide an estimate of neutral variation for comparison with immune receptor and mediating genes. The microsatellite data came from the same individuals genotyped at mediating genes in Bollmer *et al.* (2011).

Each locus had 8 to 26 alleles and all loci were in Hardy-Weinberg equilibrium with no evidence for linkage between any loci (Johnson *et al.* 2003; Bollmer *et al.* 2011).

Genetic variation and population size

To test for the effects of drift, we assessed the relationship between genetic variation and population size for each marker type (i.e., immune receptor, mediating, and microsatellites) using generalized linear models in JMP Pro v. 11 (SAS Institute 2013). We expected a positive correlation between genetic diversity and population size due to the effect of drift. We tested for differences in the effect of drift on different markers by comparing overlap in the 95% CI of the slopes for each marker across population sizes. In these analyses, we used a mean-centered estimate of genetic diversity because the three types of markers differ in polymorphism (mean alleles/locus varied from 4.7 for mediating genes to 15.5 for MHC class II) and this also helped to normalize the data. To calculate the mean-centered estimate for microsatellites and mediating genes, we divided the mean number of alleles/locus at a particular population by the mean from all populations (for that marker). For MHC genes, which involved only one or two loci, we used the total number of alleles at class I and class II in each population divided by the mean across all populations (12.8 and 15.5, respectively). Population size estimates for the six prairie-chicken populations were from census data collected in the late 1990s (Svedarsky *et al.* 2000).

Population differentiation and isolation by distance

To investigate the role of neutral and selective processes on MHC and mediating genes, we compared population differentiation at immune genes relative to neutral microsatellites. We assessed population differentiation using standardized G'_{ST} (Hedrick 2005) and Jost's *D* (Jost

2008), because the magnitude of F_{ST} is dependent on heterozygosity, which differs between our genes (i.e., microsatellite loci, MHC and mediating immune genes; Meirmans & Hedrick 2010). Pairwise G'_{ST} values were estimated by dividing the original G_{ST} values by the maximum G_{ST} value, which controls for different levels of heterozygosity between the genetic markers. We could not determine G'_{ST} at MHC class II because heterozygosity was unknown, so we also calculated Jost's D which uses the effective number of alleles (Jost 2008). We calculated G'_{ST} and Jost's D using GenAlEx (Peakall & Smouse 2006) for mediating genes and MHC class I (all single locus). To calculate Jost's D for MHC class II, we used the program SPADE (Chao & Shen 2010), and coded each allele as present or absent (i.e., a dominant marker). For microsatellites, we used the program diveRsity (Keenan *et al.* 2013) in R (R Core Team 2014) to obtain Jost's D values and associated 95% CI by bootstrapping over loci (1000 permutations). Since the general patterns were the same for the two population differentiation indices, we present the results for Jost's D below and in Table S2.1 (see Tables S2.2 and S2.3 for analyses using pairwise G'_{ST}).

To differentiate between neutral and selective processes on MHC and mediating genes, we compared population differentiation (Jost's D) at immune and neutral genes using isolation-by-distance (IBD) analyses that controlled for both differences in isolation (geographic distance) and drift (population size). Under neutrality, population differentiation should increase linearly with geographic distance between populations due to reduced dispersal between populations that are farther apart. Thus, paired comparisons of differences in IBD slopes between immune genes and neutral microsatellites from the same populations are often thought to reflect the influence of selection. For example, if selection favors similar alleles across populations or there is high gene flow across populations, then we may detect weak or no IBD (flat slopes) at immune genes

54

compared with neutral microsatellites. Conversely, stronger IBD (steeper slopes) at immune genes than microsatellites may arise if differences in selective pressures are correlated with geographic distance between populations. For example, in the case of MHC genes, populations farther apart may be more likely to have different pathogens and, thus, selection may favor different MHC alleles in those populations. We tested for IBD at immune genes and microsatellites by regressing Jost's *D* values on the straight-line distance (log10-transformed km) between population pairs using Mantel tests (1000 permutations) in IBDWS 3.16 (Jensen *et al.* 2005). This analysis does not control for the influence of drift, which can vary between different genetic markers, particularly the MHC (Eimes *et al.* 2011, Sutton *et al.* 2011). Thus, we also performed IBD analyses for each marker while controlling for differences in size between population pairs using partial Mantel tests. Analyses of IBD use repeated samples of the same population, so we tested for differences between types of markers by comparing the 95% CI of the slopes for each marker.

Results

MHC diversity

We found a total of 25 and 30 alleles at class I and II, respectively. At both MHC class I and II, larger populations generally had a greater number of alleles and theta k, an index of allelic richness. Private alleles made up 25% (14/55) of the total MHC alleles, and each population had at least one private MHC allele (Table 2.1). The larger populations in Kansas and Nebraska had the most private alleles (3 and 4, respectively), while the small and isolated populations in Wisconsin and Texas each had two private alleles each. At MHC class I, heterozygosity was high across all populations (0.81-0.91), and there was no excess of heterozygotes (HWE tests).

Genetic variation and drift

Genetic diversity (mean-centered) increased with population size (log10-transformed) at all marker types, consistent with the effects of drift (Figure 2.1), but the rate of increase differed between markers (interaction $\chi^2 = 10.2$, df = 3, $P = 0.018$). The relationship had greater support in a quadratic (AIC = -13.1) than linear model (AIC = -2.6). As population size increased, MHC variation increased at a greater rate (slope [CI], MHC I: 0.300 [0.105 - 0.495]; MHC II 0.313 [0.204 - 0.423]) than variation at either neutral microsatellites (0.178 [0.021 - 0.335]) or mediating genes (0.121 [0.002 - 0.240]). These results were influenced by the small, isolated population in TX, as excluding it from the analysis led to a non-significant difference in slope between genes (marker and population size interaction: $\chi^2 = 6.53$, df = 3, $P = 0.088$; marker effect: $\chi^2 = 3.22$, df=3, $P= 0.359$), but the effect of population size remained ($P < 0.001$). Genetic diversity at both MHC and mediating genes was correlated with variation at microsatellites (all $r > 0.92$, $P < 0.01$, in analyses with all six populations and also when excluding TX), providing more evidence of drift influencing these genes. However, in regression analyses of each immune gene in relation to microsatellite variation, the slope [95% CI] of the relationship was significantly steeper for MHC class I (1.5 [1.08 - 2.02]) than mediating genes (0.7 [0.50 - 0.84]), and nearly significant for MHC Class II (1.4 [0.59 - 2.21]). Results were qualitatively similar after excluding TX from the analyses.

Population differentiation and isolation by distance

MHC genes exhibited stronger population differentiation (Jost's *D*) than both microsatellites and mediating immune genes, consistent with local adaptation (Figure 2.2, Table S2.1). For MHC genes, the majority of class I (73%, 11/15) and class II (60%, 9/15) pairwise Jost's *D* values were

above the 95% confidence intervals for microsatellites. In contrast, the majority (60%, 9/15) of Jost's D comparisons for mediating genes fell below the 95% confidence intervals for microsatellites.

MHC genes also showed stronger isolation by distance (IBD) than both microsatellites and mediating genes (Figure 2.3A, Table 2.2, Table S2.3). The IBD slopes for MHC class I ($b = 1.216$, 95% CI: 0.776–1.656) and class II ($b = 1.137$, 95% CI: 0.606–1.661) were steeper than the slopes for microsatellites ($b = 0.276$, 95% CI: 0.149–0.403) and the mediating genes ($b = 0.301$, 95% CI: 0.129–0.471; Figure 2.3A). IBD at mediating genes did not differ from that of microsatellites when all five genes were included. However, one of the mediating genes (IAP-1) was previously identified as an outlier in terms of local adaptation (i.e., it had a greater slope; Bollmer *et al.* 2011). When IAP-1 was excluded from this analysis, the IBD slope for the remaining four immune genes was significantly lower than that of microsatellites ($b = 0.120$, 95% CI 0.055–0.186; Table 2.2), and Jost's D values were not correlated with geographic distance (Table 2.2).

Drift could also contribute to differences between the IBD slopes of MHC and mediating genes. In particular, the steeper IBD slope for the MHC may have been influenced by including Texas (Figure 2.2), which has the smallest and most isolated population. However, excluding Texas had no qualitative effects on the IBD analyses, as the slopes remained steeper at MHC class I ($b = 0.628$, 95% CI: 0.221–1.034) and class II (b = 0.797, 95% CI: 0.160–1.433), compared to mediating genes ($b = 0.150$, 95% CI: 0.036–0.264; Figure 2.3B). Furthermore, when we controlled for differences in population size (including TX) with partial Mantel tests, there was still significant IBD at both MHC class I (partial $r_M = 0.794$, $P = 0.010$) and class II (partial $r_M = 0.712$, $P = 0.028$), but not at mediating genes (partial $r_M = 0.419$, $P = 0.171$).

Microsatellites also continued to show IBD after controlling for population size differences (partial $r_M = 0.650$, $P = 0.033$).

Discussion

Understanding the effects of contemporary selection and drift at functionally different immune genes is becoming increasingly important as concern intensifies about pathogen outbreaks and their potential negative impact on wild populations (Radwan *et al.* 2010; Siddle *et al.* 2007; Smith *et al.* 2009). As predicted, we found strikingly different patterns of variation between immune-receptor and mediating genes in prairie-chicken populations. In particular, there was stronger population differentiation at immune-receptor genes of the MHC than at mediating genes or neutral microsatellites, which likely results from a combination of drift and selection for locally adapted MHC genes. In contrast, mediating genes exhibited weaker population differentiation than microsatellites, suggesting that selection favors similar allele frequencies across populations despite the effects of drift. Immune-receptor (MHC) genes appeared to be affected more strongly by drift than mediating genes, which is consistent with previous studies of the effects of bottlenecks on MHC variation (Ejsmond & Radwan 2009; Eimes *et al.* 2011). Overall, our results are consistent with the hypothesis that local pathogens play a stronger role in shaping variation at genes involved in recognizing specific pathogens than genes that are involved in other immune functions.

Few studies have directly compared immune-receptor and mediating genes to investigate how selection shapes variation across populations. In these cases the results have been mixed, particularly when comparing immune-receptor genes of the innate immune system (toll-like receptors; TLRs) and mediating cytokines. For example, one study of humans found weak differentiation between populations at both TLR and cytokine genes (Ferrer-Admetlla *et al.*

2008), while another study of the same immune genes (but different populations) showed stronger differentiation at TLRs than cytokines (Ryan *et al.* 2006). Interestingly, Ryan *et al.* (2006) found that pairwise F_{ST} values for 15 mediating genes spanned two orders of magnitude (0.002 – 0.268), similar to this study (F_{ST}: 0.000 – 0.182). This large range of population differentiation at mediating genes suggests that selection can vary widely within this functional group.

In prairie-chickens, we found stronger differentiation among populations at MHC genes than at four (including three cytokines) of the five mediating genes. One mediating gene (IAP-1) had stronger population differentiation, similar to MHC. Most of this effect was driven by a unique polymorphism in IAP-1 found within the Wisconsin population (Bollmer *et al.* 2011). It is unknown whether the polymorphism within IAP-1 has any fitness-related effects in prairie-chickens, but in domestic chickens a single nucleotide polymorphism within this gene had different effects on chick body weight in environments with high and low hygiene (Ye *et al.* 2006), suggesting that selection may vary depending on environmental conditions. Intracellular pathogens are known to disrupt apoptosis by inhibiting the production of mediating molecules (e.g., cytokines), and, as a result, prevent an appropriate immune response (Seow 1998; Alcami 2003). Thus, some mediating genes involved in apoptosis, such as IAP-1, may interact directly with pathogens, which could lead to local adaptation.

Similar to several other studies, we found stronger population differentiation at MHC than neutral loci, suggesting that differences in local selection pressures are important (Ekblom *et al.* 2007; Kyle *et al.* 2014; Oliver *et al.* 2009). However, there are exceptions to this pattern, including studies of other grouse. For example, black grouse (*Tetrao tetrix*) in Europe have weaker differentiation at MHC class II than microsatellite loci across populations of different

size (50 to >1000 birds) and isolation (200-2000 km between populations), suggesting that balancing selection favors similar alleles across populations (Strand *et al.* 2012). These differences between studies could arise if greater gene flow of advantageous MHC alleles (relative to neutral genes) leads to less differentiation of MHC at large geographic scales, while local adaptation of MHC alleles at smaller geographic scales (relative to neutral genes) produces stronger differentiation (Hederegen *et al.* 2014). However, contrary to this hypothesis, MHC differentiation was stronger in prairie-chickens and weaker in black grouse, even though the sampling scales were similar (maximum distances between populations were similar: ~2000 km). An alternative explanation may be that pathogen-mediated selection at the MHC differs between these species of grouse. Indeed, several studies have found that MHC variation across populations is related to differences in pathogen prevalence or strains, even within the same species, consistent with this hypothesis (Prugnolle *et al.* 2005; Bonneaud *et al.* 2006; Dionne *et al.* 2007; Tollenaere *et al.* 2008; Bichet *et al.* 2015).

These inferences about the role of selection in shaping variation at immune genes need to be viewed with some caution as drift also influenced variation at both MHC and mediating genes and our sample of populations was small (Figure 2.1). In particular, the effect of drift seemed to be stronger on MHC than other genes when we analyzed all six populations, but not when we excluded Texas, which has the smallest and most isolated population. However, MHC genes still exhibited a steeper IBD slope than both mediating genes and microsatellites when we removed the Texas population from the IBD analyses (which reduced the effect of drift), Thus, it appears that selection is contributing to stronger MHC differentiation among populations despite potentially stronger effects of drift on MHC than other genes (Eimes *et al.* 2011, Sutton *et al.* 2011). One explanation for the greater effect of drift on MHC genes is that MHC allele

frequencies are skewed as a result of negative frequency-dependent selection (i.e., rare allele advantage), and in this case, drift is likely to remove the rare MHC alleles first, resulting in a relatively faster loss of genetic variation (Sutton *et al.* 2011). However, contrary to this hypothesis, MHC alleles in larger populations of prairie-chickens did not have a more skewed distribution than small populations (Figure S2.1), and so it is unlikely that drift alone explains the greater loss of MHC variation. Instead, a combination of drift and selection could be responsible. In particular, Ejsmond and Radwan (2009) proposed that following a bottleneck, selection may favor MHC alleles that provide resistance to current pathogens, but as these alleles increase in frequency they also expose relatively more alleles that are at low frequency to drift, and the net effect is that selection actually speeds up the loss of overall diversity. To help disentangle these effects of drift and selection, we recommend future studies use a greater number of non-bottlenecked populations (to reduce the confounding effects of drift) or examine how variation at the MHC and other immune genes changes with geographic variation in pathogen communities (e.g., Dionne et al. 2007, Eizaguirre et al. 2012).

Overall, our results suggest that both selection and drift play important roles in shaping immune gene variation, but their strength can be strikingly different between immune receptor and mediating genes. With the exception of humans, our study is the first to compare variation at immune genes from different functional groups across natural populations. We found effects of drift (population size) and isolation (IBD) on both categories of immune genes, which cautions against ascribing the type of selection acting on genes without knowledge of demography. From a conservation perspective, this study also demonstrates the need to examine a wider array of immune genes to assess adaptive variation in declining populations. While many studies have relied solely on MHC genes, it is becoming increasingly apparent that other types of immune

61

receptors (TLRs) and cytokines are associated with disease resistance (Tschirren *et al.* 2013; Turner *et al.* 2011) and survival (Grueber *et al.* 2013) in wild populations. Thus, more detailed studies of the fitness effects of particular alleles are needed to better understand how selection influences variation in different functional groups of immune genes.

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Figure 2.1. Number of alleles in relation to population size (log10-transformed) across six prairie-chicken populations. Lines are from quadratic regressions (see text). Number of alleles was averaged across loci for mediating genes and microsatellites. Total number of alleles was used for MHC class I and II. These estimates for all four markers were then centered by their means (see Methods).
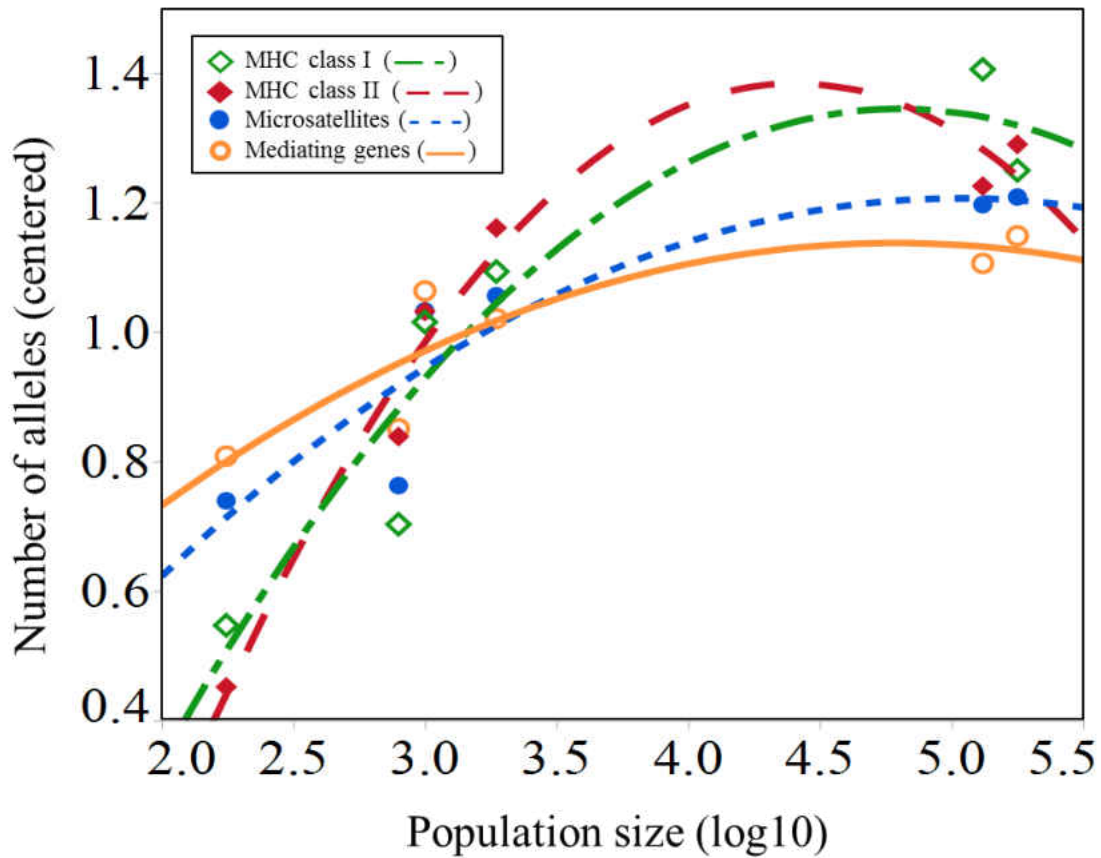
Figure 2.2. Population pairwise Jost's *D* values for six microsatellites, MHC (class I and II, separately), and five mediating genes. Error bars indicate 95% confidence intervals for the microsatellites.
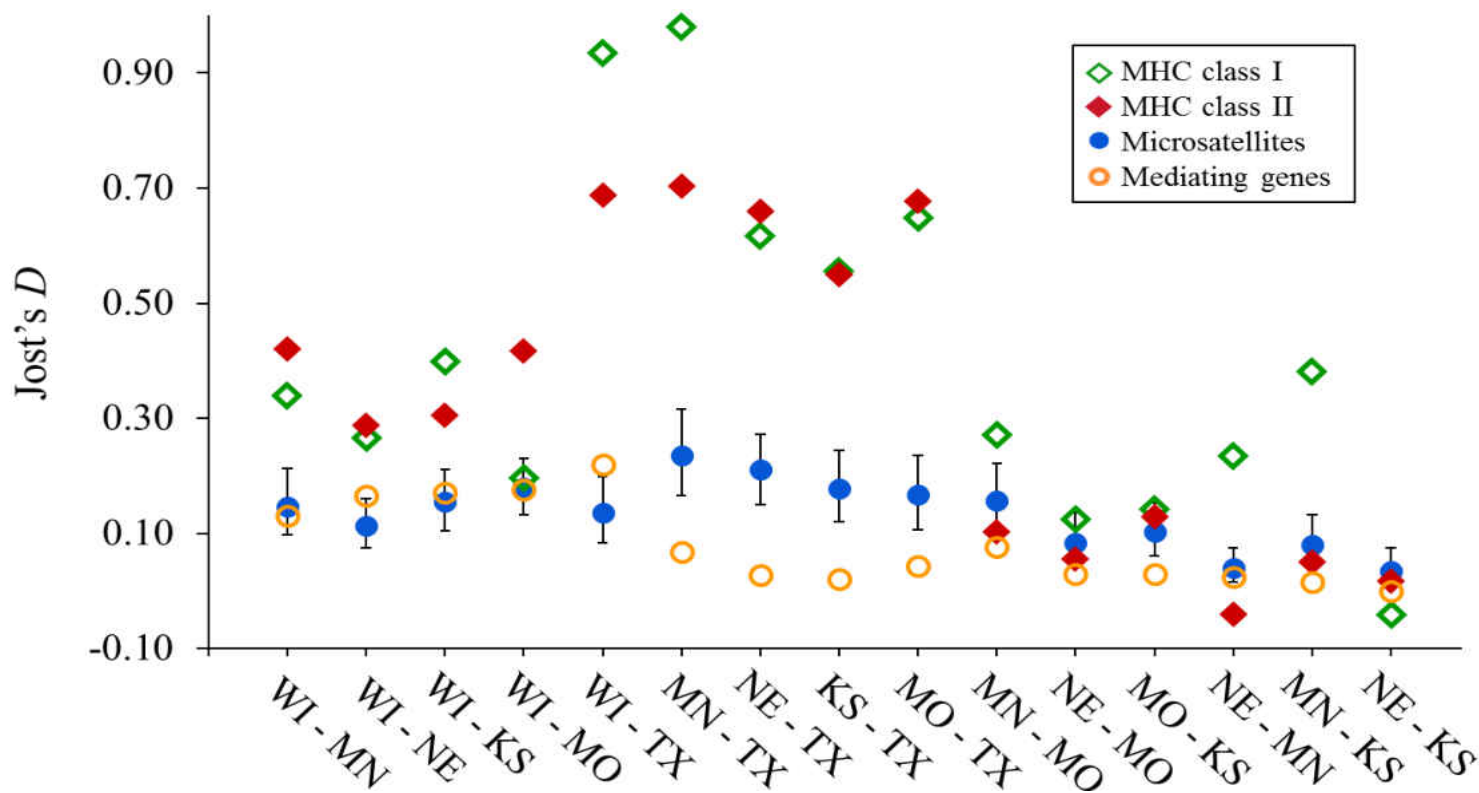
Figure 2.3. Isolation by distance in six prairie-chicken populations (A) and in five populations after excluding the small population in Texas (B). Genetic differentiation (Jost's *D*) increased at a faster rate with geographic distance (log10[km]) for MHC than the four mediating genes (excluding IAP-1) in both A and B (see text for slopes and CI).
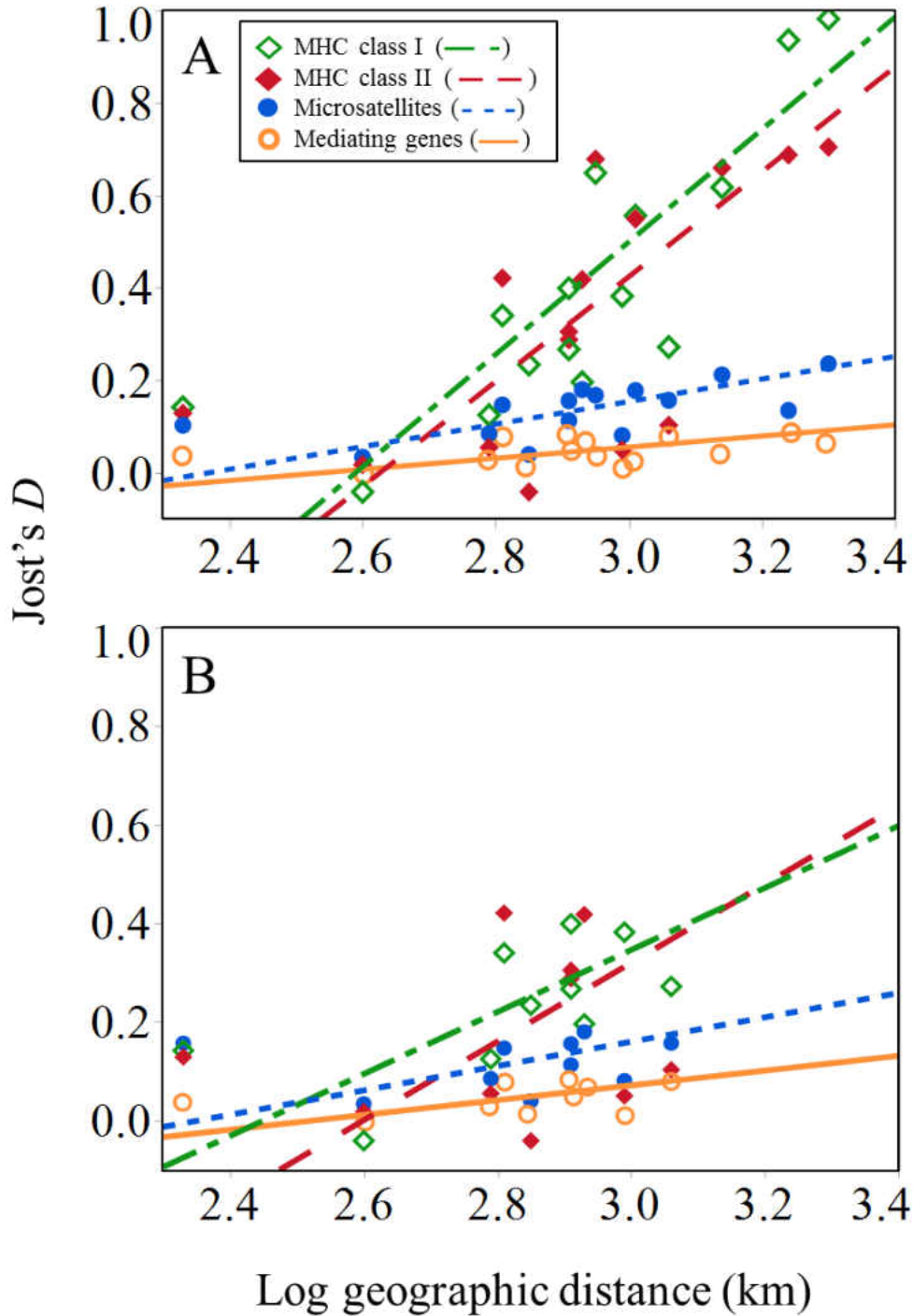
Figure S2.1. Allele frequency distributions for each of the two immune-receptor (MHC class I and II) genes separated by population. Alleles are presented in order of descending frequency (left to right) within populations. Skewness coefficients from JMP (SAS Institute, 2013) are given for each population.

MHC class I
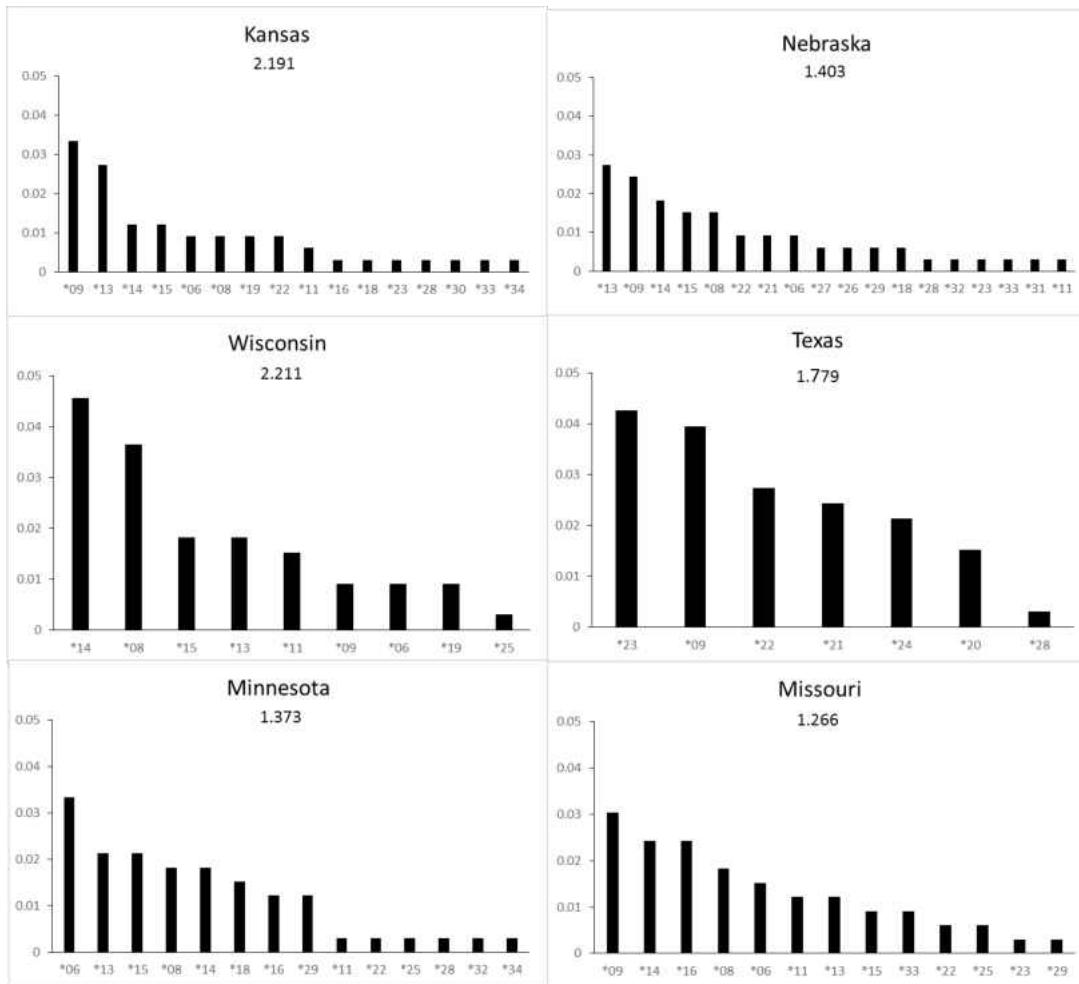
Figure S2.1. (continued)

<u>MHC class II</u>
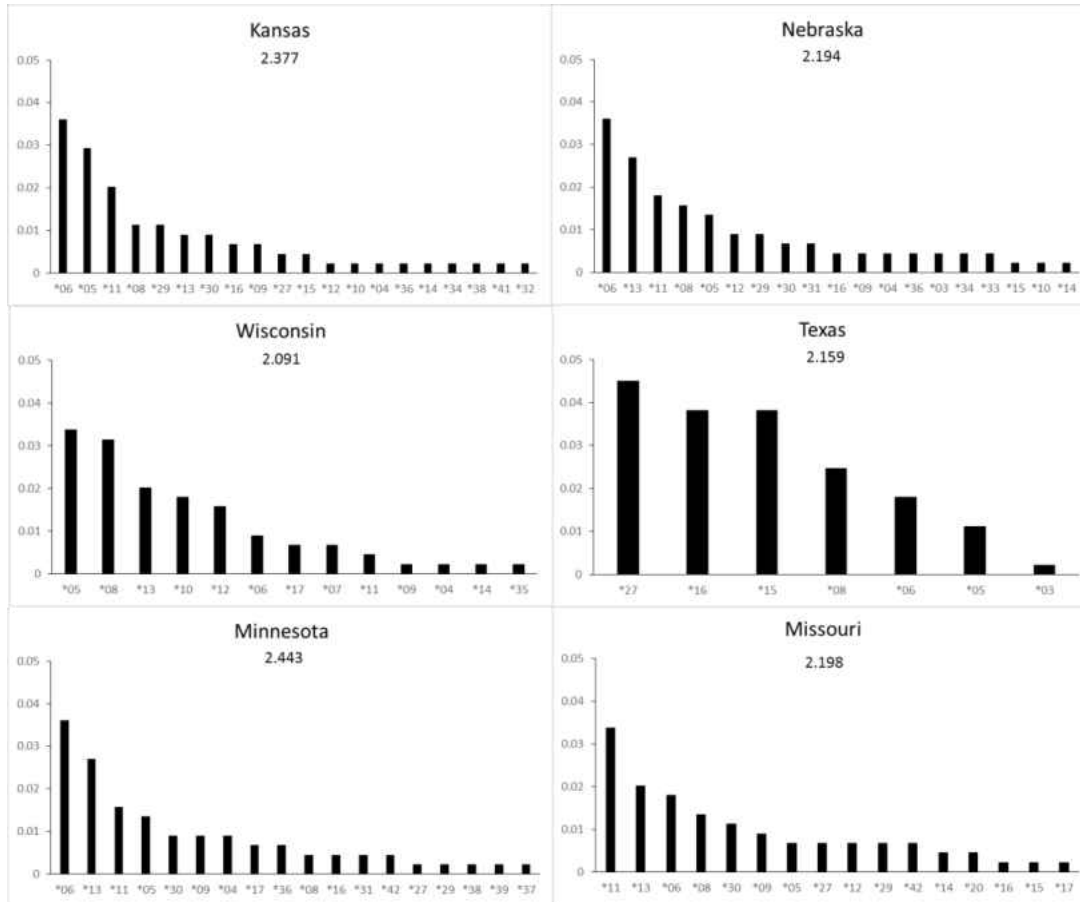
Table 2.1. Summary of MHC class I and II diversity for greater prairie-chicken populations in Kansas (KS), Nebraska (NE), Minnesota (MN), Missouri (MO), Wisconsin (WI) and Texas (TX). Census sizes were estimated by Svedarsky *et al.* (2000). Summary statistics include the number of individuals genotyped (n), number of MHC alleles (A_N) with number of private alleles in parentheses, expected heterozygosity (H_E) for class I, mean number of alleles per individual at class II (A_N/I), nucleotide diversity (π), and theta k (k).

Pop	Census size	n	MHC class I				MHC class II			
			A_N	H_E	π	k	A_N	A_N/I	π	k
KS	178000	30	16 (1)	0.86	0.041	7.86	20 (2)	2.50	0.081	8.59
NE	131484	30	18 (3)	0.91	0.042	8.79	19 (1)	2.66	0.087	7.57
MN	1868	30*	14 (0)	0.88	0.042	5.67	18 (2)	2.48	0.086	7.38
MO	1000	30	13 (0)	0.89	0.044	4.96	16 (1)	2.30	0.088	6.24
WI	794	30	9 (0)	0.82	0.034	2.83	13 (2)	2.30	0.072	4.47
TX	176	33	7 (2)	0.81	0.043	1.88	7 (0)	2.39	0.094	1.67

*n = 29 for MHC class II

Table 2.2. Isolation by distance among prairie-chicken populations. Results of Mantel tests in IBDWS using pairwise Jost's *D* values. For mediating immune genes, isolation by distance was first tested with all five genes combined (A) and then again excluding the outlier IAP-1 gene (B).

Genetic marker	Z	*r*	*P*	slope	lower 95% CI	Upper 95% CI
MHC class I	18.47	0.797	0.004	1.216	0.776	1.656
MHC class II	15.25	0.629	0.047	1.137	0.606	1.661
Microsatellites	6.17	0.641	0.021	0.276	0.149	0.403
Mediating genes (A)	3.59	0.317	0.203	0.301	0.129	0.471
Mediating genes (B)	2.07	0.419	0.846	0.120	0.055	0.186

Table S2.1. Geographic distance (km) between six prairie-chicken chicken populations and their pairwise Jost's *D* values for MHC class I and II genes, five mediating genes and six microsatellites loci. Significant comparisons (P < 0.05) are indicated in bold.

Population pairs	Distance (km)	MHC class I	MHC class II	Mediating genes	Microsatellites
WI-MN	647	**0.340**	**0.421**	**0.131**	**0.147**
WI-NE	821	**0.267**	**0.288**	**0.166**	**0.113**
WI-KS	807	**0.399**	**0.305**	**0.171**	**0.156**
WI-MO	860	**0.196**	**0.418**	**0.177**	**0.180**
WI-TX	1754	**0.935**	**0.687**	**0.219**	**0.135**
MN-TX	1978	**0.981**	**0.704**	**0.068**	**0.236**
NE-TX	1370	**0.617**	**0.659**	**0.027**	**0.212**
KS-TX	1016	**0.556**	**0.549**	**0.021**	**0.178**
MO-TX	895	**0.648**	**0.678**	**0.043**	**0.168**
MN-MO	1148	**0.272**	0.103	**0.077**	**0.157**
NE-MO	613	**0.125**	0.055	**0.029**	**0.085**
MO-KS	213	**0.142**	0.129	**0.030**	**0.103**
NE-MN	700	**0.234**	-0.041	**0.025**	0.040
MN-KS	980	**0.382**	0.050	0.016	**0.081**
NE-KS	400	-0.041	0.018	-0.001	0.034

Table S2.2. Pairwise G'_{ST} values between six prairie-chicken populations at MHC class I, five mediating genes and six microsatellites loci between six prairie-chicken populations. Significant comparisons ($P < 0.05$) are indicated in bold.

Population pairs	MHC class I	Mediating	Microsatellites
WI-MN	**0.358**	**0.169**	**0.219**
WI-NE	**0.280**	**0.224**	**0.150**
WI-KS	**0.418**	**0.230**	**0.212**
WI-MO	**0.208**	**0.236**	**0.206**
WI-TX	**0.941**	**0.302**	**0.221**
MN-TX	**0.982**	**0.106**	**0.266**
KS-TX	**0.577**	**0.039**	**0.221**
MO-TX	**0.665**	**0.058**	**0.205**
NE-TX	**0.634**	**0.046**	**0.232**
MN-MO	**0.282**	**0.096**	**0.169**
NE-MO	**0.130**	**0.038**	**0.103**
MO-KS	**0.149**	**0.041**	**0.106**
NE-MN	**0.244**	**0.033**	**0.031**
MN-KS	**0.397**	**0.018**	**0.078**
NE-KS	-0.044	-0.001	**0.015**

Table S2.3. Isolation by distance among prairie-chicken populations. Results of Mantel tests in IBDWS using pairwise G'_{ST} values. For mediating genes isolation by distance was tested with all five genes combined (5 genes) and excluding the outlier IAP-1 gene.

Genetic Marker	Z	r	P value	Slope	Lower 95% CI	Upper 95% CI
MHC class I	18.96	0.798	0.010	1.220	0.780	1.661
Microsatellites	7.28	0.633	0.029	0.323	0.173	0.473
Mediating genes (5 genes)	4.89	0.351	0.195	0.403	0.177	0.629
Mediating genes (no IAP)	2.80	0.438	0.090	0.161	0.074	0.248

Specific alleles at immune genes, rather than genome-wide heterozygosity, are related to immunity and survival in the critically endangered Attwater's prairie-chicken

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Abstract

The negative effects of inbreeding on fitness are serious concerns for populations of endangered species. Reduced fitness has been associated with lower genome-wide heterozygosity and immune gene diversity in the wild; however, it is rare that both types of genetic measures included in the same study. Thus, it is often unclear whether the variation in fitness was due to the general effects of inbreeding, immunity-related genes, or both. Here, we tested whether genome-wide heterozygosity (20 990 SNPs) or diversity at nine immune genes were better predictors of two measures of fitness (immune response and survival) in the endangered Attwater's prairie-chicken (*Tympanuchus cupido attwatteri*). We found that post-release survival of captive-bred birds was related to alleles of the innate (toll-like receptors, TLRs) and adaptive (major histocompatibility complex, MHC) immune systems, but not to genome-wide heterozygosity. Likewise, we found that the immune response at the time of release was related to TLR and MHC alleles, and not to genome-wide heterozygosity. Overall, this study demonstrates that genetic information can potentially help improve survival rates for captive-release programs, and that specific functional genes may be better predictors of fitness than robust genome-wide heterozygosity in severely inbred populations.

Introduction

Many small and isolated populations experience reduced fitness, presumably due to the effects of low genetic diversity and inbreeding (Keller and Waller 2002). Inbreeding increases homozygosity, which can lead to reduced fitness (inbreeding depression), primarily through the expression of recessive deleterious alleles (Charlesworth and Willis 2009; Kristensen, et al. 2010). Many studies have examined the effects of inbreeding on fitness by correlating fitness with indices of inbreeding from genetic markers such as microsatellites (Hansson and Westerberg 2002; Chapman, et al. 2009). Since these are putatively neutral markers, it is often assumed that correlations between fitness and heterozygosity are caused by linkage between the neutral markers and functional loci that affect fitness (Kristensen, et al. 2010; Szulkin, et al. 2010). Studies using heterozygosity-fitness correlations (HFC) have been controversial, because in the past they have generally relied on a small number of markers (<30 microsatellite loci; Chapman, et al. 2009). As a consequence, a lack of correlation between neutral markers and fitness could be due to inadequate genome-wide sampling and weak linkage with functionally important loci (Chapman, et al. 2009). The number and choice of markers will also limit our ability to determine if inbreeding depression is caused by loss of heterozygosity at a small number of functionally important loci or more widespread homozygosity across the genome. As a result, there have been calls for more studies of the effects of inbreeding on heterozygosity (Kristensen, et al. 2010; Szulkin, et al. 2010) and gene expression (Hansson, et al. 2014; Menzel, et al. 2015) at functional loci directly related to fitness.

In terms of fitness effects, genes of the immune system, such as the major histocompatibility complex (MHC) and toll-like receptors (TLR), have been among the most studied. MHC genes code for receptor molecules that are involved in activating an adaptive

(specific) immune response by recognizing antigens from specific pathogens (Janeway, et al. 2005). Many studies of the MHC have found associations with immune response (Bonneaud, et al. 2005; Charbonnel, et al. 2010; Cutrera, et al. 2011), pathogen resistance (Langefors, et al. 2001; Arkush, et al. 2002; Kloch, et al. 2010; Osborne, et al. 2015) and survival (Worley, et al. 2010; Dunn, et al. 2013). Fewer studies have explored the effects of innate immune genes on disease-related traits in wild populations, even though they may be responsible for more of the genetic diversity related to disease resistance than the MHC (Jepson, et al. 1997). For example, TLRs activate an immediate, innate (non-specific) immune response by detecting conserved features of large groups of pathogens (Medzhitov 2001). There is growing evidence that TLR diversity (Hartmann, et al. 2014) or specific TLR genotypes (Grueber, et al. 2014) are linked to survival in small inbred populations. Although these studies provide evidence for the importance of particular immune loci to fitness, it is largely unknown whether the gene-fitness relationships arise from the effects of the immune genes themselves or are a consequence of a broader, more general effect of inbreeding (e.g., reduced heterozygosity across the genome). Thus, to gain a more comprehensive assessment of the relationship between fitness and inbreeding, it will be important to include both functional loci, such as immune genes, and larger scale estimates of genome-wide heterozygosity.

The captive breeding program of the Attwater's prairie-chicken (*Tympanuchus cupido attwatteri*) provides a rare opportunity to study the relationship between fitness and heterozygosity at both immune genes and across the genome. Attwater's prairie-chicken populations have declined dramatically, and, as a consequence, have lost genetic variation at both microsatellites (Hammerly, et al. 2013) and a variety of immune genes (Bollmer, et al. 2011; Bateson, et al. 2015). To prevent their extinction in the wild, a captive population of

80

Attwater's prairie-chickens was established in the early 1990s from approximately 19 unrelated founders (U.S. Fish & Wildlife Service 2010). Each year since 1995, the wild population has been supported by the release of 150-200 captive-bred birds, which are radio-tagged and monitored daily. However, both survival of chicks in captivity ($\leq 66\%$; Morrow, et al. 2004) and released juveniles (~20% range: 8-43%; Pratt 2010) is low due partially to high levels of relatedness between mated pairs (Hammerly, et al. 2015). The intensive management and daily monitoring of released birds gives us detailed estimates of individual survival that are rarely found in other studies, except other captive-release programs (e.g., fish hatcheries) or in populations where dispersal events are very limited (e.g., isolated islands).

In this study, we asked whether heterozygosity across the genome or at particular immune genes is a better predictor of immune response and post-release survival. We predicted that birds with higher genome-wide heterozygosity would have higher rates of survival because inbreeding appears to negatively influence the survival of chicks in the captive population during the first few weeks after hatching (Hammerly, et al. 2013). Since certain alleles may be more important than heterozygosity *per se* at immune genes, we also investigated whether there were allele-specific relationships with post-release survival. To test these predictions, we estimated genome-wide heterozygosity with >20,000 single nucleotide polymorphisms (SNPs) and allelic variation at nine immune genes. We also examined the relationship between immune response and genetic variation, both at immune loci and genome-wide. The cloacal microbiota of captive and wild Attwater's prairie-chickens differ in their abundance of gram positive and negative bacteria (Simon 2014), so we included two assays that examined the innate immune response against gram positive and gram negative bacteria (lysozyme and bacteria killing assays, respectively).

Methods

For this study, we sampled a total of 144 Attwater's prairie-chickens that were released in 2011 ($n = 67$) and 2012 ($n = 77$). These birds originated from five captive-breeding facilities in Texas, which includes the Abilene Zoo ($n = 16$), Caldwell Zoo ($n = 2$), Fossil Rim Wildlife Center ($n = 79$), Houston Zoo ($n = 37$) and San Antonio Zoo ($n = 9$). The average (±SE) age of birds chosen for release was 245 ±24 days and did not differ between birds released in 2011 (261 ±35 days) and 2012 (231 ±32 days; $t = -0.63$, df $= 142$, $P = 0.53$). Prior to release, birds were examined by a veterinarian and those considered healthy were transferred from the breeding facilities to the Attwater's Prairie Chicken National Wildlife Refuge [N 29° 39' 46.786", W 96° 16' 56.249"] near Eagle Lake, TX (Colorado County). At the refuge, birds were held in acclimation pens (soft release) for 14 days before release into the wild (Lockwood, et al. 2005). We monitored the birds every day through radio-tracking to obtain the number of days alive in the wild until 15 September 2015 when the last of the radio-tracked birds died (see Morrow, et al. 2015 for more details).

Blood preparation for Plasma and DNA Extraction

We collected approximately 300 µl of blood from the jugular vein of each bird before they were placed in the acclimation pens. This sample was immediately transferred to a heparinized vial, stored on ice for 1-2 hours and then centrifuged at 9300g for 10 min to separate plasma from the red blood cells. Plasma was stored at -80 °C for later use in the immunological assays. We stored the remaining red blood cells in Queen's lysis buffer (Seutin, et al. 1991) until we extracted DNA (DNEasy Tissue Extraction Kit, Qiagen Inc).

Genome-wide heterozygosity

We used double-digest restriction-site-associated DNA sequencing (ddRADseq) to identify single nucleotide polymorphisms (SNP), which were then used to estimate genome-wide heterozygosity of individuals. ddRAD libraries were prepared following the protocol of Peterson, et al. (2012), and all libraries were prepared and sequenced at Texas A&M AgriLife Genomics Facility. Briefly, genomic DNA was digested with the restriction enzymes SphI and MluCI. Barcode adapters (110 bp) were ligated, and then fragments in the 260-340 bp size range were size-selected using a Pippen Prep instrument (Sage Science). Amplified fragment libraries were quantified and then pooled in equimolar amounts for sequencing on six lanes of an Illumina HiSeq 2500 machine (100 bp paired-end reads). While the six lanes included samples from multiple projects, the initial ddRADseq data set (*n* = 130) used in this study consisted of 282,498,476 paired demultiplexed raw sequence reads.

The *dDocent* pipeline (Puritz, et al. 2014) was used for quality trimming (Trimmomatic v.0.33; Bolger, et al. 2014), read mapping (BWA-MEM v.0.7.12; Li and Durbin 2010), and SNP calling (Freebayes v.0.9.20; Garrison and Marth 2012). Default parameters were used for each step, with the exception of using a high coverage greater prairie-chicken (*T. c. pinnatus*) reference genome for read mapping as opposed to a *de novo* reference contig assembly, and only paired-end reads were retained following the initial trimming step using Trimmomatic. The reference genome used for mapping was sequenced and assembled by Dovetail Genomics (www.dovetailgenomics.com; Santa Cruz, CA) at 44x depth of coverage resulting in 12,364 scaffolds with an N50 of 12.2 Mb.

Further filtering using VCFTools v.1.11 (Danecek, et al. 2011) and custom scripts was done to retain those SNPs that met several quality and population genetic criteria: genotyped in at least 90% of all samples, a minimum quality score of 30, minor allele count of at least 3, and a minor allele frequency >5% across the dataset. One sample possessed >50% missing SNPs, and was subsequently removed from the dataset. Additional filtering using scripts available with the *dDocent* package (https://github.com/jpuritz/dDocent) were used to remove possible paralogs or repetitive regions within the dataset (see also Portnoy, et al. 2015). Loci were excluded if the average allele balance at heterozygous genotypes was less than 28%, had a quality score less than half of the total depth, the ratio between the mean mapping quality of the reference and alternate allele was less than 0.9 or more than 1.05, possessed overlapping and improper paired forward and reverse reads, had a depth greater than the average depth plus one standard deviation if the quality score was less than 2x the depth, and were in the top 10% of mean depth. Only those loci that conformed to expectations of Hardy Weinberg Equilibrium (HWE) with a *P*-value threshold of 0.01 were retained for further analysis. The final dataset contained 20 990 biallelic SNPs in 129 birds with an average sequencing depth of 56x per individual (range = 24 to 235x). To obtain heterozygosity values for the final SNP dataset, the program PLINK v. 1.07 (Purcell, et al. 2007) was used to create a binary coded output file (--recode12), which was then used to calculate standardized heterozygosity (SH) for each individual in the R package Rhh (Alho, et al. 2010). This measure of heterozygosity controls for variation in heterozygosity across loci and accounts for missing genotypes (Coltman, et al. 1999).

MHC class I and II

We genotyped individuals at MHC class I (exon 3) and MHC class II (exon 2) loci, which code for the peptide-binding regions (PBRs) that recognize pathogens. For MHC class I, we used primers 1a2inExon3F and 1a2Raltintron2 with PCR conditions described in Bateson, et al. (2014). There was a 3 bp sequence length polymorphism at position 40 in exon 3 and sequences varied in length from 258 bp (most common) to 261 bp (Bateson, et al. 2015). To amplify MHC class II (exon 2) we used primers Blex2F (Eimes, et al. 2010) and RNA R1a (Strand, et al. 2007) that produced a 237-bp fragment (for PCR conditions see, Eimes, et al. 2010). Prairie-chickens have a single class I locus and two class II loci (Eimes, et al. 2013); therefore, similar to our previous work (Bateson, et al. 2015), we expected to amplify maximum of two (class I) and four (class II) sequences within individuals.

Due to the complexity at MHC class I (sequence length polymorphism) and class II (multiple loci), we used 454 pyrosequencing to genotype individuals, which used fusion primers containing Roche 454 adapter sequences, a unique 8-bp barcode (to identify individuals) and either MHC class I or class II primers. MHC class I and class II PCR products from each individual (amplicons) were purified, pooled and sequenced on a Roche 454 FLX Genome Sequencer with Titanium chemistry at Research and Testing Laboratory, LLC (Lubbock, TX).

MHC allele and genotyping validation

We performed several quality control steps to separate true sequences (hereafter alleles) from artifacts, which are known to occur in MHC sequences from 454 pyrosequencing (for details see, Bateson, et al. 2015). Briefly, we first extracted sequences that contained the forward primer, 8-

bp barcode and 10 bases of the reverse primer using jMHC v. 1.5 (Stuglik, et al. 2011) and removed sequences that were >2 bp from expected sizes of exons. Next, we clustered the remaining sequences by building neighbor-joining trees in Geneious (version 7.0, http://www.geneious.com, Kearse, et al. 2012). Each cluster contained a high read sequence (the true allele) and low read sequences with 1-2 bp errors due to homopolymer runs (typically $\leq 5\%$ of the total reads of the true allele). The number of reads from the low read sequences were added to the final read count of the true alleles. To identify and discard any remaining artifact sequences and determine the number of alleles within individuals, we used the two independent models of Lighten, et al. (2014). We also assessed the repeatability of our genotyping procedure by resequencing a subset of prairie-chickens in a second 454 pyrosequencing run (Bateson, et al. 2015).

After our filtering and processing steps, we had an average ($\pm$SE) of 176 $\pm$ 17 (range: 5 – 1255) reads per individual at class I, and 1432 $\pm$ 139 (range: 141 – 13,035) reads per individual at class II. To assess whether we had sufficient sequencing coverage, we used the binomial distribution to calculate the minimal number of reads needed to find all alleles within individuals (amplicons) 95% of the time, given a maximum of two class I and four class II alleles per individual. Assuming equal probability of finding each allele (0.5 at class I; 0.25 at class II), we estimated that we would need a minimum of 5 class I reads and 11 class II reads to find all alleles within individuals. For class I, there were just two individuals at the 5 reads threshold and both were heterozygous (i.e., had two alleles). For class II, the minimum number of reads in an individual (141) was well above the 11 read threshold. Additionally, there were no correlations between total number of reads and alleles at class I (r^2= 0.002, $F_{1,138}$ = 0.321, P = 0.572) or class II (r^2= 0.001, $F_{1,139}$ = 0.20, P = 0.649), indicating sufficient sequencing coverage for genotyping

in our sample. We had high repeatability (R=0.93; $F_{35, 36}$ = 29.13; $P < 0.001$) in our 454 class I

and class II genotyping for 18 prairie-chickens (Bateson, et al. 2015), which included five

Attwater's prairie-chickens used in this study.

Single-locus immune genes

We initially screened 16 additional immune gene loci for polymorphism, which included

receptors (toll-like receptors and natural killer receptors), cytokines and β. These genes were

chosen because they have polymorphisms associated with either fitness-related traits, or previous

studies found signatures of selection (dN/dS > 1) at the sequence level. After our initial screening

in prairie-chickens (see Tables S1 and S2 for PCR conditions and methods), we focused on seven

genes that contained nonsynonymous polymorphisms (Table 3.1). First, we investigated TLR

variation within the extra-cellular regions (leucine-rich repeat domain) that are involved in

pathogen recognition (Bell, et al. 2003). Here, we investigated four loci (TLRs 1B, 4, 5, and 15)

that detect a variety of cellular components of bacteria or fungi (Table 3.1). Next, we examined

the C-type lectin-like receptor gene (Blec1), which is found within the MHC region of galliform

birds, including prairie-chickens (Eimes, et al. 2013). While the function of Blec1 is largely

understudied in birds, it has high amino acid similarity to CD69 (Shiina, et al. 2007; Rogers and

Kaufman 2008), an early activation antigen within the natural killer gene family. In humans,

CD69 may be important in regulating an inflammatory immune response, and some allelic

variants are associated with chronic inflammatory diseases (González-Amaro, et al. 2013). We

also examined the cytokine interleukin 4 (IL4) gene, which is a signaling molecule critical for

mediating humoral (antibody) activity of the adaptive immune responses (Borish and Steinke

2003). Particular IL4 haplotypes have been associated with reduced risk of malaria and other diseases in human populations (Jha, et al. 2012). Lastly, we examined diversity at β –defensin 11, which are catatonic peptides that disrupt cell walls of bacteria (Ganz 2003), and in great tits (*Parus major*), peptide products from different alleles vary in their ability to suppress bacterial growth *in vitro* (Hellgren, et al. 2010).

For each of these seven single-locus immune genes, PCR products were directly sequenced in both directions at the University of Chicago Cancer Research Center DNA Sequencing Facility. We performed a BLAST search to confirm that sequences matched the targeted gene and then aligned them with the domestic chicken (*Gallus gallus*) genome to identify exons and introns. We considered SNPs within individuals when there were heterozygous peaks in both the forward and reverse sequences. Once all individuals were sequenced, alleles were determined using the PHASE algorithm (Stephens and Donelly 2003) in DNAsp v5 (Librado and Rozas 2009) with 1000 main iterations, a thinning interval of 10 and 1000 burn-in interactions. For IL4, approximately half of the individuals contained sequence length polymorphisms due to an allele-specific indel at position 153 within intron 1. Therefore, before phasing IL4 the direct sequence traces of individuals with heterozygous indels were reconstructed using the default settings of the program In*d*elligent (Dmitriev and Rakitov 2008). All DNA sequences of immune genes were retained if sequences were found in at least two individuals (i.e., two independent PCRs). For all subsequent analyses, we removed introns (sequenced at Blec1, IL4 and BD11) and combined DNA sequences that had identical amino acid sequences into the same allele. Complete immune gene sequences (i.e., including introns) were submitted to NCBI GenBank.

Single-locus immune gene genetic diversity estimates

Since there could be specific or general effects of genetic diversity on fitness, we assessed genetic diversity at immune genes in terms of: 1) presence of particular alleles, 2) heterozygosity at particular loci and 3) standardized (average) heterozygosity across loci. First, specific alleles may be better predictors of immune response and survival instead of heterozygosity, so we tested the presence or absence of immune gene alleles for each individual. By examining alleles, we were able to include the multi-locus data from MHC class II and, thus, reduce the total number of analyses. In these models we included the six immune genes with >2 alleles each (TLR1B, TLR15, IL4, Blec1, MHC class I and II) and, for each gene, we excluded alleles that occurred in $\leq$ 5% of the sampled birds. Second, we examined the effects of heterozygosity at specific immune genes by coding individuals as homozygote (0) or heterozygote (1) at each gene, respectively. Lastly, we examined the general effects of heterozygosity across all single-locus immune genes (MHC class II excluded) by measuring standardized heterozygosity (SH) in the R package Rhh.

Immunological assays

Bacterial killing assay

We used a bacteria-killing assay to measure the ability of plasma to lyse gram negative bacteria *in vitro*, predominately through natural antibodies and complement (Millet, et al. 2007; Liebl and Martin 2009). We followed a modified protocol of Millet, et al. (2007). Briefly, we diluted *Escherichia coli* (ATCC no. 8739; American Type Culture Collection, Manassas, VA) lyophilized pellets to a working stock solution of 1×10^5 CFU/mL. Next, we mixed 1.5 μL of

plamsa with 34.5 µL of PBS and 12.5 µL of the *E.coli* solution (approximately 1250 CFU per sample) and incubated the mixture for 30 min at 39 °C (body temperature of Attwater's prairie-chickens). After incubation, we added 250 µL of sterile tryptic soy broth, vortexed the sample and then pipetted 50 µL of this mixture onto a tryptic soy agar plate, which was incubated at 37 °C for 12 h (optimal growing temperature of *E. coli*). After this second incubation period, we counted the number of CFU on each plate. The antimicrobial activity of plasma was calculated as 1-nb/nt, where nb is the number of surviving bacteria for each plasma sample and nt is the number of bacteria on control sample. Each plasma sample was tested three times to get an average BKA value for each bird. Three plates in each batch of 15 samples served as controls where plasma was replaced with 1.5 µL of PBS. Repeatability (Lessells and Boag 1987) between replicates of each individual was 0.94 ($n = 441$).

Haemagglutination and haemolysis assays

To measure the response of circulating natural antibodies and complement in plasma to a foreign antigen, we used a modified version of the protocol developed by Matson, et al. (2005), which consisted of two indices of innate immunity. The first index, haemagglutination, measures the interaction between natural antibodies and foreign antigens (in our case, rabbit erythrocytes). The second index measures haemolysis, which estimates the activity of complement from the levels of haemoglobin released during the lysis of rabbit erythrocytes. For these assays, 20 µL of both plasma and PBS was pipetted into the first well of each row of a 96-well plate, and then 1:2 serial dilutions were made in wells 2-11 using PBS. The remaining well (12) was used as a negative control by adding only PBS. Next, we added 20 µL of 1% rabbit erythrocytes

suspension to each well and incubated the plates at 37 °C for 90 min. Following incubation, the

plates were tilted at a 45° angle for 20 min to improve visibility of agglutination before they

were scanned on an Epson perfection v100 flatbed scanner at 300 dpi. After an initial scan, the

plates were kept at room temperature for an additional 70 min and scanned a second time to

assess the maximum lytic activity. As suggested by Matson, et al. (2005), plates were scored

blindly for agglutination and lysis by a single observer (SCH). Repeatability between replicates

of each individual was 0.80 ($n = 390$) for the haemaggluintation assay and 0.53 ($n = 434$) for the

haemolysis assays.

Lysozyme assay

Lysozyme is a protective protein of the innate immune system that hydrolyzes linkages in the

cell walls of gram positive bacteria. We indirectly measured lysozyme concentrations in plasma,

by using a modified protocol of Meier, et al. (2013). Briefly, using 96-well plates, 10 µL of each

plasma sample was run in duplicate in 150 µL of a bacterial solution that consisted 0.01 g of

Micrococcus lysodeikticus (ATCC 4698; Sigma-Aldrich, St. Louis, MO) in 20 mL of 1% sterile

agarose. To quantify lysozyme levels for each plasma sample, we used a standard curve

prepared on each plate in quadruplicate from a serial dilution (0.039 to 2.5 mg/L) of a standard

domestic chicken lysozyme solution (Sigma L6876) using sterile 0.1M sodium phosphate (pH

6.2). Both plasma and sodium phosphate blanks were also included in duplicate on each plate,

and the final prepared plate was incubated at 49 °C for 18 h before reading the absorbance (850

nm) using a microplate reader (Synergy HT, Bio-Tek, Winooski, VT). Repeatability between

replicates of each individual was 0.95 ($n = 671$).

<u>Statistical analyses</u>

We used mixed models to analyze immune response and survival in relation to genome-wide and immune gene diversity. In the allele-specific models, there were 28 immune gene alleles that met our initial criteria for predictor variables (i.e., genes with >2 alleles and alleles found in $\geq$ 5% of sampled birds), which included alleles at MHC class I ($n = 6$), MHC class II ($n = 6$), Blec1 ($n = 5$), TLR1B ($n = 4$), TLR15 ($n = 4$) and IL4 ($n = 3$). In the heterozygosity models, we included measures of standardized heterozygosity from the 8 single-locus immune genes (excludes MHC class II) or heterozygosity of individual loci. We also included genome-wide standardized heterozygosity, body mass (at entry into pens), sex, and family group (as a random effect) in each of the allele-specific and heterozygosity models. For each immune response and survival model, we checked for predictors with high multicollinearity as indicated by variance inflation factors (VIF) and removed variables that had VIF > 2 (Neter, et al. 1996) from further analyses using JMP 12 Pro (SAS Institute 2015). Only the allele-specific models had predictors with VIFs > 2, and, removing these predictors resulted in the same 18 immune gene alleles across models. Due to the large number of predictors, we also calculated q values, which are estimates of the proportion of false discoveries for all P values within each model, using a FDR of 0.10 and the graphically sharpened method of Pike (2010).

Immune response

We performed four separate generalized linear mixed models (GLMM) with the response from each of the four immunoassays as the dependent variable. The fixed predictors in these models were immune gene heterozygosity or specific alleles, genome-wide heterozygosity (ddRAD-

seq), sex and body mass (g); family group was included as a random factor. All analyses were conducted in JMP 12 Pro.

Survival

To examine the effects of genetic diversity on post-release survival, we used Cox proportional hazard mixed effect models in the R package coxme (Therneau 2012). Cox models estimate the baseline hazard function where predictor estimates (exp(β)) with no effects on the hazard (e.g., death) are equal to 1 and any categorical variable (e.g., presence/absence of an allele) with parameter estimates >1 increase the hazard of death while estimates <1 increase the likelihood of survival. Since missing data in the predictor variables can lead to biased interpretations in survival analyses, we only used birds ($n = 116$) with complete genetic data. We obtained data on survival (days until death) for 110 birds; an additional six birds were included in the analysis but were censored to the last day observed before they went missing ($n = 4$) or experienced radio failure ($n = 2$). All survival models included genome-wide heterozygosity, sex, body mass at release (g) as predictors and family group as a random factor. For immune gene predictors, we performed three Cox models to examine the effects of standardized heterozygosity, single-locus heterozygosity and specific alleles, separately.

Results

After release from the pens, the median survival for birds was 133 days (interquartile range: 33 - 217 days; $n = 110$). There was no difference in survival between birds released in 2011 (median 133 days, interquartile range 36 – 222 days; $n = 54$) and 2012 (median 158 days; $n = 56$; interquartile range 36 – 286 days; log-rank $X^2 = 0.98$, df = 1, $P = 0.320$). Genome-wide standardized heterozygosity averaged 0.994 (range: 0.834 – 1.615). In contrast to our prediction,

93

we found no relationship between survival and genome-wide standardized heterozygosity (at 20 990 SNPs), indicating no apparent effects of inbreeding on post-release survival ($q = 0.782$, Table 3.2). Likewise, immune response prior to release was not associated with genome-wide heterozygosity in the allele models ($q > 0.1$ across models; Table 3.3 and S3.4).

Immune genes and survival

We found that survival was related to six alleles from five immune genes as well as body mass, while controlling for family ID (Cox proportional mixed model: $X^2 = 37.83$, df = 21, $P = 0.014$; Table 3.2). Survival was not related to year (Z= -0.23, df = 1, $P = 0.820$), so it was not included in the model (Table 3.2). All alleles associated with survival were pathogen-recognition receptor genes from the innate (TLRs) and adaptive (MHC) immune systems. Interestingly, alleles from these two different categories of receptors had opposite relationships with survival. Birds with TLR1B*03 or TLR15*02 had lower survival rates, while birds with particular MHC alleles (class I: Tycu-IA*22 and Tycu-IA*24; class II: Tycu-BLB*08; and MHC-linked Blec1*04) had higher survival rates (Table 3.2). The MHC class II allele (Tycu-BLB*08) exhibited the strongest association with survival (Table 3.2, Figure 3.1). Only one allele (Tycu-IA*22) was related to both immune response (lower lysozyme activity) and survival (increased survival). Along with the presence of specific MHC alleles, individuals with greater body mass survived longer after release. In contrast, survival was not related to standardized heterozygosity averaged across the eight single-locus immune genes ($P = 0.82$, $n = 116$; excludes MHC class II) or to heterozygosity at each individual locus ($P \geq 0.19$; Table S3.3).

Immune genes and immune response

Immune response was also related to specific alleles rather than heterozygosity at immune genes. We found that the innate immune response, as measured by the lysozyme and haemolysis assays, was related to alleles at four immune genes as well as to body mass and sex (Table 3.3). Specifically, we found that TLR1B*02, Tycu-IA*22, and Blec1*01 were associated with reduced lysozyme activity against gram positive bacteria. Males also had stronger lysozyme activity than females. A different MHC class I allele (Tycu-IA*20) and lower body mass was associated with reduced haemolysis activity. Across all models, immune response as measured by the bacterial killing with agglutination assays was not related to immune genes, body mass or sex (see Table S3.4 and S3.5).

Discussion

The detrimental effects of inbreeding on disease resistance are serious concerns for wild and captive populations of endangered species. While studies have demonstrated that individual fitness is related to genome-wide heterozygosity and immune gene diversity, rarely are both types of genetic measures included in the same study. Thus, it is unknown whether variation in fitness was due to the general effects of inbreeding, key immunity-related genes, or both. In the critically endangered Attwater's prairie-chicken, we found that post-release survival of captive-bred birds was related to specific alleles of the innate (TLR) and adaptive (MHC) immune systems, and not related to genome-wide heterozygosity. These results contrast with those from younger birds, in which chicks with reduced microsatellite heterozygosity had reduced survival shortly after hatch in the captive population (Hammerly, et al. 2013). We also found that the

immune response near the time of release was related to specific alleles of the innate and adaptive immune systems, and not to genome-wide heterozygosity. Overall, these results suggest that specific alleles of immune genes are better indicators of immune response and survival than genome-wide heterozygosity.

*Genome-wide heterozygosity, immune respon*se *and survival*

Reduced genome-wide heterozygosity has been associated with reduced survival (Coltman, et al. 1999; Townsend, et al. 2010) and immune response (Hawley, et al. 2005), suggesting that inbreeding influences disease resistance in wildlife populations. However, there are other studies that do not find correlations between genetic diversity and measures of fitness. For example, disease-related mortality was not related to genome-wide heterozygosity in captive-released bighorn sheep (*Ovis canadensis*; Boyce, et al. 2011) or a semi-natural population of red jungle fowl (*Gallus gallus*; Worley, et al. 2010). The absence of correlations between genetic diversity and measures of fitness may be due to a number of factors, including a relatively small number of genetic markers (10-30 loci) that reduced statistical power (Chapman, et al. 2009). In our study, however, genome-wide heterozygosity was estimated from 20 990 SNPs, suggesting that the absence of correlations between genetic diversity and measures of fitness was not due to an insufficient number of loci.

Perhaps a more important factor affecting the power of HFC studies is variation in the extent of inbreeding among individuals within the study population, i.e., the effect size (Grueber, et al. 2008; Szulkin, et al. 2010). For example, severely inbred nestlings had weaker innate immune responses and higher disease-related mortality as juveniles in a study of American

crows (*Corvus brachyrhynchos* (Townsend, et al. 2009). However, this relationship between heterozygosity and survival disappeared when the highly inbred crows (coefficient of relatedness ≈ 0.5) were removed from the analysis (Townsend, et al. 2010). In captive Attwater's prairie-chickens, chicks produced by more closely related parents had lower survival during the first two weeks post-hatch and juvenile and adult birds with high inbreeding coefficients have lower survival in captivity (Hammerly, et al. 2013). In our study, the released birds had already reached an average age of 245 ±24 days, and they had to pass several health checks before release. Thus, there was already some survival selection for birds at earlier life stages in captivity, which may have reduced variation in genome-wide heterozygosity in the released cohort.

Immune genes and survival

Associations between MHC genes and survival have been found in wild (Paterson, et al. 1998; Sepil, et al. 2013) and captive (Penn, et al. 2002; Kjøglum, et al. 2008) populations, but only a few studies have investigated whether these associations are independent of genome-wide variation. For example, MHC heterozygotes had higher survival after exposure to an infectious virus in Chinook Salmon (*Oncorhynchus tshawytscha*), which was not explained by the level of inbreeding within families (Arkush, et al. 2002). In the threatened New Zealand sea lion (*Phocarctos hookeri*), a specific MHC class II genotype and allele were associated with pup survival that was not related to microsatellite heterozygosity (Osborne, et al. 2015). In our study of Attwater's prairie-chickens, specific alleles (not heterozygosity) at the MHC were related to increased post-release survival. In particular, the MHC class II allele, Tycu-BLB*08, had the strongest effect on survival. During the study period, birds with this allele were 3.5 times more likely to survive than those without it. Interestingly, the presence of a specific Blec1 allele also increased the likelihood of survival. In prairie-chickens, Blec1 is located within the compact

97

MHC region (Eimes, et al. 2013), and, while, functionally different than the MHC class I and II genes, it might play a role in regulating a MHC-related immune response (González-Amaro, et al. 2013). Overall, our results are consistent with an increasing number of theoretical (De Boer, et al. 2004; Ejsmond and Radwan 2015) and empirical (reviewed in Sin, et al. 2014) studies that suggest a stronger role of frequency dependent or fluctuating selection at the MHC; in this case specific alleles (not heterozygosity) confer a selective advantage and influence fitness.

Pathogens have been associated with specific alleles and diversity at cytokines (Turner, et al. 2011) and TLRs (Tschirren, et al. 2013) in wild populations, suggesting that other immune genes, in addition to MHC, may be important to individual survival. For instance, reduced survival during migration was associated with elevated expression of cytokines (interleukins) in threatened Sockeye salmon (*Oncorhynchus nerka*) that were infected with viral pathogens (Jeffries, et al. 2014). In contrast to most HFC studies, greater heterozygosity at TLRs was associated with lower survival in the endangered Pale-headed Brush finch (*Atlapetes pallidiceps*; Hartmann, et al. 2014). However, in this study, the authors did not explicitly test for links between specific TLR alleles and survival. In our study, there were no associations between survival and heterozygosity at cytokine IL4 or TLRs, but we found two TLR alleles (TLR1B*03 and TLR15*02) that were related to lower survival. Disadvantageous alleles at immune genes have been reported elsewhere, as certain MHC alleles are associated with increased risk of infection in birds (Bonneaud, et al. 2006; Loiseau, et al. 2008), mammals (Srithayakumar, et al. 2011) and fish (Langefors, et al. 2001; Kjøglum, et al. 2008). Persistence of these alleles is usually explained through antagonistic pleiotropy, in which alleles have opposing effects on fitness under different circumstances (Carter and Nguyen 2011), such as exposure to different pathogens (Loiseau, et al. 2008). In our particular case, the appearance of disadvantageous

alleles could be a product of the differences between the captive and wild environments. Captive and wild Attwater's prairie-chickens appear to have different cloacal microbiomes (Simon 2014), which might interact with the two TLR alleles to affect survival differently in the wild and captivity. Testing this hypothesis will require more complete data on cloacal microbes, TLR alleles and survival in both environments.

Immune genes and immune response

Vertebrate studies of immune genes and immunity have mainly focused on associations between MHC diversity and an induced immune response. Measuring induced immunity of individuals requires multiple recaptures and injections of foreign antigens, such as phytohemagglutinin (PHA) or sheep red blood cells (SRBC). Responses to these antigens have been linked to both specific alleles and heterozygosity at the MHC (Bonneaud, et al. 2005; Charbonnel, et al. 2010; Cutrera, et al. 2011). Unlike the PHA and SRBC assays, our indices of immunity were measured from a single capture, and, therefore, evaluate the naturally circulating immunological components that provide an immediate and non-induced immune response (e.g. lysozyme and complement). We found specific immune gene alleles associated with only two (lysozyme and haemolysis) of the four assays, indicating the limitations of using a single immune assay to generalize non-induced immunity (Adamo 2004; Forsman, et al. 2008).

Interestingly, we found both MHC and TLR alleles that were associated with a reduced immune response, particularly lysozyme activity. Lower lysozyme activity has been associated with lower disease-related mortality in salmon populations (Lund, et al. 1995; Balfry, et al. 1997). Therefore, TLR and MHC alleles associated with lower lysozyme activity might indicate higher

fitness in captive Attwater's prairie-chickens possessing those alleles. In fact, one MHC allele (Tycu-IA*22) was associated with both lower lysozyme activity and increased survival in the wild. However, previous work did not find an association between lysozyme activity and post-release survival in a larger sample of Attwater's prairie-chickens ($n = 192$; Hammerly 2014), and so it is not clear how these negative relationships are produced between immune gene alleles and the non-induced immune response.

In summary, we found that the immune response of Attwater's prairie-chickens and their subsequent survival in the wild were related to alleles of both the innate (TLR) and adaptive (MHC) immune systems, and not to genome-wide heterozygosity. This suggests that pathogens in the environment may substantially impact the survival of captive-bred birds released into the wild. However, we do not know the specific cause of death (i.e., disease or predation) of the released Attwater's prairie-chickens in this study, and thus, the relative role of pathogens in the wild and captivity is still unclear. In the future, it will be important to investigate how variation at these immune genes influences mortality. For example, examining associations between immune genes and microbial diversity in the cloaca of both captive and wild birds could reveal important clues to the host-pathogen interactions that might be influencing survival (e.g., Bolnick, et al. 2014). Overall, our study illustrates that genetic information can potentially help improve survival rates for captive-release programs, and that in some cases at least, specific immune genes may be better predictors of fitness than genome-wide heterozygosity. As captive-breeding programs are being used increasingly to supplement the remaining populations of endangered species, understanding the genetic (Grueber, et al. 2015) and ecological (Morrow, et al. 2015) factors that are driving recruitment success will be necessary to ensure population persistence in the wild.

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Figure 3.1. Post-release survival of Attwater's prairie-chickens in relation to MHC class II allele, Tycu-BLB*08. Kaplan-Meier plot shows that birds with Tycu-BLB*08 had higher proportional survival than birds without the allele (n = 116, log-rank: X^2=5.60, P=0.02).
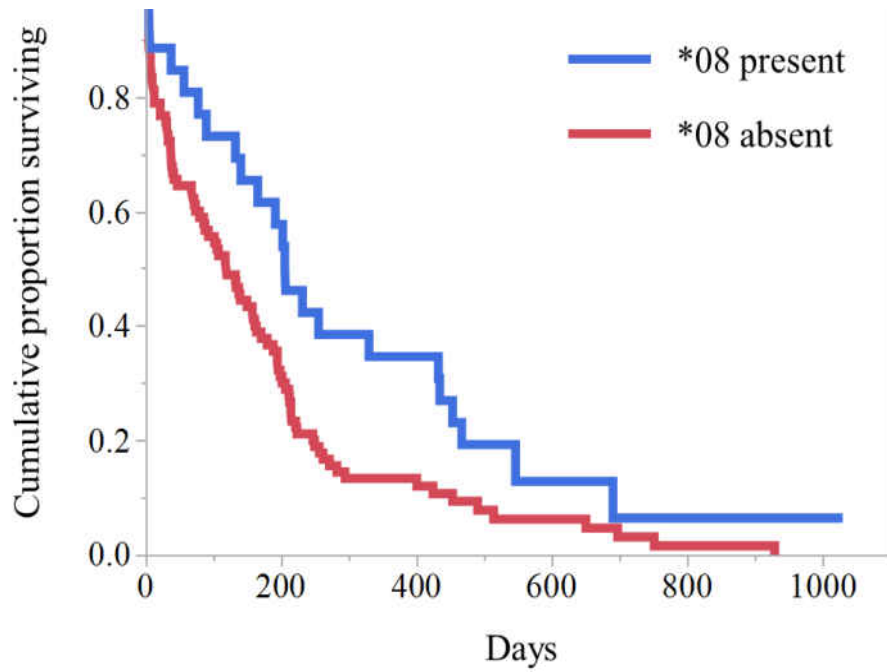
Table 3.1. Summary of immune gene diversity in Attwater's prairie-chickens. Included are the number of birds (n), particular exons sequenced and the corresponding length in base pairs (intron length in parentheses if amplified), number of nonsynonymous SNPs (NS), and number of alleles (A_N).

Immune gene	Function	n	Exon(s)	Sequence length	NS	A_N
MHC region						
MHC class I	Recognizes intracellular pathogens	143	3	258-261	16	7
MHC class II	Recognizes extracellular pathogens	141	2	239	36	8
C-type lectin-like receptor 1 (Blec1)	Early activation antigen	139	3,4	223 (534)	3	5
Non-MHC						
Toll-like receptor 1B (TLR1B)	Recognizes cell wall of bacteria and fungi	142	1	284	3	4
Toll-like receptor 4 (TLR4)	Recognizes LPS	143	3	787	1	2
Toll-like receptor 5 (TLR5)	Recognizes flagellin	139	1	1222	1	2
Toll-like receptor 15 (TLR15)	Recognizes fungi	144	1	597	3	4
Interleukin 4 (IL4)	B and T cell growth factor	144	1,2	178 (327)	3	4
β-Defensin 11 (BD11)	Disrupts bacterial membranes	137	2	131 (188)	1	2

Table 3.2. Post-release survival of Attwater's prairie-chickens ($n = 116$) in relation to specific alleles at immune genes, genome-wide heterozygosity, body mass (g), sex and Family ID (random factor). Presented are the parameter estimates (β), standard error (SE), hazard ratio ($Exp(\beta)$) and P values from the Cox proportional mixed model. q values are the probability (P) values corrected for the false discovery rate (FDR).

Predictor	β	SE	Exp(β)	*P*	*q*
Genome-wide heterozygosity	-0.091	0.954	0.913	0.920	0.782
MHC class I					
Tycu-IA*20	-0.324	0.462	0.723	0.480	0.544
Tycu-IA*22	-0.741	0.305	0.477	**0.015**	**0.048**
Tycu-IA*24	-0.741	0.310	0.476	**0.017**	**0.048**
MHC class II					
Tycu-BLB*08	-1.262	0.351	0.283	**<0.001**	**0.005**
Blec1					
Blec1*01	0.082	0.329	1.086	0.800	0.756
Blec1*02	0.107	0.330	1.113	0.750	0.750
Blec1*04	-0.931	0.330	0.394	**0.005**	**0.040**
TLR1B					
TLR1B*01	0.259	0.329	1.296	0.430	0.522
TLR1B*02	0.062	0.380	1.064	0.870	0.778
TLR1B*03	0.662	0.284	1.938	**0.020**	**0.049**
TLR1B*04	-0.007	0.308	0.993	0.980	0.793
TLR15					
TLR15*01	0.711	0.576	2.035	0.220	0.340
TLR15*02	0.861	0.355	2.365	**0.015**	**0.048**
TLR15*03	0.317	0.399	1.373	0.430	0.522
TLR15*04	0.495	0.448	1.641	0.270	0.383
IL-4					
IL4*01	0.323	0.262	1.382	0.220	0.340
IL4*02	-0.665	0.530	0.514	0.210	0.340
IL4*03	0.153	0.456	1.166	0.740	0.750
Body mass (g)	-0.002	0.001	0.998	**0.017**	**0.048**
Sex	0.534	0.262	1.706	**0.042**	0.089

Table 3.3. Immune response of Attwater's prairie-chickens in relation to specific alleles at immune genes, genome-wide heterozygosity, body mass (g), sex, and family group (as a random factor). Presented are the parameter estimates (β), standard error (SE), *P* and *q* values for each predictor variable in the models for Lysozyme (*n* = 115) and Haemolysis (*n* = 114). The models for bacteria-killing and agglutination assays are in the supplemental material (Table S3.4).

Predictor	Lysozyme				Haemolysis			
	β	SE	*P*	*q*	β	SE	*P*	*q*
Genome-wide heterozygosity	0.362	0.207	0.086	0.183	-0.790	0.577	0.175	0.458
MHC class I								
Tycu-IA*20	-0.037	0.130	0.777	0.690	-1.070	0.291	<0.001	**0.004**
Tycu-IA*22	-0.316	0.090	0.001	**0.007**	-0.270	0.193	0.166	0.458
Tycu-IA*24	-0.212	0.102	0.040	0.136	0.007	0.216	0.974	0.975
MHC class II								
Tycu-BLB*08	0.035	0.105	0.743	0.690	-0.353	0.234	0.135	0.458
Blec1								
Blec1*01	-0.307	0.112	0.007	**0.031**	0.153	0.225	0.498	0.747
Blec1*02	-0.001	0.098	0.995	0.805	-0.341	0.205	0.100	0.458
Blec1*04	-0.019	0.077	0.812	0.690	-0.260	0.195	0.186	0.458
TLR1B								
TLR1B*01	-0.177	0.096	0.070	0.170	-0.274	0.221	0.218	0.458
TLR1B*02	-0.340	0.114	0.004	**0.023**	0.319	0.254	0.213	0.458
TLR1B*03	0.035	0.088	0.689	0.689	-0.058	0.186	0.756	0.934
TLR1B*04	-0.042	0.091	0.643	0.684	0.106	0.215	0.623	0.817
TLR15								
TLR15*01	-0.184	0.171	0.285	0.484	-0.295	0.369	0.427	0.690
TLR15*02	-0.052	0.102	0.616	0.684	0.008	0.243	0.975	0.975
TLR15*03	-0.073	0.116	0.529	0.684	0.131	0.247	0.598	0.817
TLR15*04	0.056	0.102	0.580	0.684	-0.044	0.251	0.860	0.950
IL-4								
IL4*01	-0.042	0.075	0.580	0.684	-0.187	0.168	0.269	0.513
IL4*02	-0.150	0.222	0.501	0.684	0.622	0.370	0.099	0.458
IL4*03	-0.198	0.118	0.100	0.188	-0.071	0.313	0.822	0.950
Body Mass (g)	0.000	0.000	0.062	0.170	0.003	0.001	<0.001	**0.002**
Sex	-0.168	0.036	<.0001	**0.002**	-0.083	0.080	0.302	0.528

Table S3.1. Summary of the other single-locus immune genes initially surveyed in a subset of prairie-chickens. Included are the number of birds (*n*), particular exons sequences and the corresponding length in base pairs (intron length in parentheses if amplified, number of synonymous (Syn) and intron SNPs. Note that none of these immune genes had nonsynonymous SNPs.

Immune gene	*n*	Exon(s)	Sequence length	Syn SNPs	Intron SNPs
Toll-like receptor 3 (TLR3)	21	4	747	1	-
Toll-like receptor 7 (TLR7)	21	2	570	1	-
Interleukin 1B (IL1B)	21	5,6	357 (607)	3	4
Interleukin 6 (IL6)	4	2,3	174 (309)	0	0
Interleukin 6 (IL6)	21	4	189	1	-
Interleukin 10 (IL10)	21	3,4	219 (728)	1	3
β-Defensin 02 (BD02)	21	2,3	177 (410)	1	3
β-Defensin 04 (BD04)	4	3	100	0	-
Lysozyme (Lyso)	21	1	136 (324)	2	3

Immune gene screening methods

We screened for single nucleotide polymorphisms (SNPs) at candidate immune genes by initially sequencing three Attwater's prairie-chickens and one greater prairie-chicken from either the genetically diverse Kansas or Nebraska population. If we found SNPs between subspecies (i.e., between Attwater's and greater prairie-chickens), we randomly selected an additional 16 Attwater's prairie-chickens to sequence. Next, we identified exons by aligning each sequence with the domestic chicken (*Gallus gallus*) genome using the BLAST algorithm in Geneious version 7.0. We retained immune genes for analysis in the entire data set if there were non-synonymous SNPs in this initial sample of 21 prairie-chickens. Overall, seven single-locus immune genes met our criteria with these screening steps.

Table S3.2. Primers and PCR conditions used to amplify the seven single-locus immune genes used in the study (A) and other immune genes that did not meet our initial screening criteria (B). Each immune gene was amplified in PCRs (final volume 20ul) consisting of 1x Green GoTaq Flexi Buffer (Promega), 1.5 mM of $MgCl_2$, 10% DMSO, 0.5 µM of each primer, 0.4 mM of dNTPs, 1.0 U of GoTaq DNA polymerase and approximately 50 ng of genomic DNA. PCRs were performed with an initial denaturation step at 94 °C for 2 min followed by 35 cycles of 20 s at 94 °C, 20 s at locus-specific annealing temperature (T_A) and specific extension time (T_E) at 72 °C.

A)

Locus	T_A (° C)	T_E (sec)	Primer names	Primer sequences (5′-3′)	References
TLR1B	59	80	avTLR1LBF	TCCAGGYTWCAAAATCTGACAC	Alcaide & Edwards 2010
			avTLR1LBR	CGGCACRTCCARGTAGATG	
TLR4	50	80	avTLR4F	GAGACCTTGATGCCCTGAG	Alcaide & Edwards 2010
			avTLR4R	CCATCTTRAGCACTTGCAAAG	
TLR5	55	80	avTLR5F	GTAATCTTACCAGCTTCCAAGG	Alcaide & Edwards 2010
			avTLR5R	GCTGGAGTTCATCTTCATC	
TLR15	62	45	TLR15F1	GCTGGGTGCTGTTTTGGAGT	This study
			TLR15R1	GAGGTGCTGCAGAGAGATCG	
Blec1	62	45	Blec1F	AGCTCCCACGTTTCTCATCC	This study
			Blec1R	CCAAGGCAAGGATGGGAACT	
IL4	54	45	IL4ex1-2F	ATGAGCTCCTCACTGCCCAC	Downing et al. 2010
			IL4ex1-2R	CTGGCTTTCCTCTTACCTTA	
BD11	60	45	AvBD11F1mat	GACTGATCCTGCAGCACAAC	Hellgren & Sheldon 2011
			AvBD11R1	AGGGCTCCCACACGTACC	

B)

Locus	T_A (° C)	T_E (sec)	Primer names	Primer sequences (5′-3′)	References
TLR3	53	80	pcTLR3Falt	ACCTCTCACTGAGCCATGTG	This study
			pcTLR3R	TGTTGTTATTGCTGATGTC	
TLR7	59	60	pcTLR7F2	GAAGCTTATCCCCAGTCTTG	This study
			pcTLR7R3	AGACAGGTAGCAGAATTCGC	
IL1B	59	60	IL1Bex5-6F	CTTCGACATCTTCGACATCAAC	Downing et al. 2009
			IL1Bex5-6R	ATACGAGATGGAAACCAGCAAC	
IL6 (a)	63	45	IL6ex2-3F	CGAGAACAGCATGGAGATGC	This study
			IL6ex2-3R	GTGGCCGCCAGGTGCTTTGT	
IL6 (b)	57	45	IL6ex4F	GTGATAAATCCCGATGAAGT	This study
			IL6ex4R	TCAGGCACTGAAACTCCTGG	
IL10	62	45	IL-10_F1	CCACTGCTGGGGTTCAGATT	This study
			IL-10_R1	CTCTCTCCCCACCCACTGAA	
BD02	55	45	AvBD2ex2F	ATGAGGATTCTTTACCTGC	This study
			AvBD2ex3R	CATTTGCAGCAGGAACGGAA	
BD04	56	45	pcBD4ex3F	TGTTCAGGCTTTCCCCGTCC	This study
			pcBD4Ralt	TCAGTTTAGCCATAGTCAAG	
Lyso	60	45	LYSOex1_Falt	GAGACAGGTGCAAGAGAGCC	This study
			LYSOex1_Ralt	GGGAAAGGAGCGTAAAGGGA	

Table S3.3. Post-release survival of Attwater's prairie-chickens (n = 116) in relation to standardize heterozygosity (A) and single-locus heterozygosity (B) at immune genes, genome-wide heterozygosity, body mass (g), and sex. In both analyses, family group was used as a random factor. Presented are the parameter estimates (β), standard error (SE), hazard ratio (Exp(β)) and corresponding P values from the Cox proportional mixed models.

A)

Predictor	β	SE	Exp(β)	P
Genome-wide het	0.128	1.136	0.882	0.88
Immune genes het	0.082	1.085	0.350	0.82
Body mass	-0.001	0.999	0.001	0.51
Sex	0.511	1.667	0.229	**0.03**

B)

Predictor	β	SE	Exp(β)	P
Genome-wide het	-0.243	0.785	0.924	0.79
MHC class I	-0.068	0.934	0.286	0.81
Blec1	-0.218	0.804	0.280	0.44
TLR1B	0.267	1.307	0.237	0.26
TLR4	0.491	1.634	0.505	0.33
TLR5	-0.413	0.662	0.450	0.36
TLR15	0.161	1.174	0.265	0.55
IL4	0.138	1.148	0.238	0.56
BD11	-0.793	0.452	0.599	0.19
Body mass	< 0.001	1.000	0.001	0.83
Sex	0.408	1.503	0.252	0.11

Table S3.4. Immune response of Attwater's prairie-chickens in relation to specific alleles at immune genes, genome-wide heterozygosity, body mass (g) and sex. Family group was used as a random factor. Presented are the estimates (β), standard error (SE), *P* and *q* values for each predictor variable in the models for Bacteria killing assay (*n* = 116) and Agglutination assay (*n* = 107).

Predictor	Bacteria killing				Agglutination			
	β	SE	*P*	*q*	β	SE	*P*	*q*
Genome-wide het	-0.397	0.357	0.269	1.017	1.739	1.127	0.128	0.670
MHC class I								
Tycu-IA*20	-0.049	0.172	0.776	1.017	-0.018	0.572	0.975	0.975
Tycu-IA*22	0.032	0.112	0.772	1.017	0.209	0.395	0.597	0.913
Tycu-IA*24	-0.158	0.124	0.208	1.017	-0.018	0.448	0.969	0.975
MHC class II								
Tycu-BLB*08	-0.072	0.136	0.599	1.017	0.218	0.471	0.645	0.913
Blec1								
Blec1*01	0.238	0.130	0.071	1.017	0.053	0.473	0.911	0.975
Blec1*02	-0.035	0.121	0.770	1.017	0.164	0.418	0.695	0.913
Blec1*04	-0.064	0.117	0.587	1.017	0.245	0.377	0.519	0.913
TLR1B								
TLR1B*01	-0.040	0.131	0.761	1.017	-0.273	0.441	0.538	0.913
TLR1B*02	0.169	0.148	0.255	1.017	0.803	0.503	0.115	0.670
TLR1B*03	-0.021	0.109	0.849	1.017	0.038	0.375	0.920	0.975
TLR1B*04	0.001	0.125	0.996	1.046	-0.252	0.437	0.566	0.913
TLR15								
TLR15*01	-0.147	0.220	0.506	1.017	0.684	0.778	0.382	0.891
TLR15*02	-0.005	0.143	0.972	1.046	-0.119	0.481	0.805	0.975
TLR15*03	0.040	0.146	0.786	1.017	-0.621	0.488	0.207	0.707
TLR15*04	0.047	0.149	0.754	1.017	0.475	0.488	0.333	0.874
IL-4								
IL4*01	-0.029	0.098	0.765	1.017	-0.549	0.337	0.108	0.670
IL4*02	0.033	0.208	0.876	1.017	-0.329	0.751	0.664	0.913
IL4*03	-0.073	0.188	0.699	1.017	-0.842	0.606	0.169	0.707
Body Mass (g)	< 0.001	< 0.001	0.686	1.017	0.001	0.001	0.236	0.707
Sex	-0.015	0.046	0.740	1.017	0.259	0.158	0.106	0.670

Table S3.5. Immune response of pre-released Attwater's prairie-chickens in relation to standardized heterozygosity (A) and single-locus heterozygosity (B) at eight immune genes, genome-wide heterozygosity, body mass (g) and sex. In these models, family group was included as a random factor. Sample sizes (*n*), parameter estimates (β), standard error (SE) and *P* values are given from the generalized linear mixed models. Single-locus heterozygosity models (B) also include false discovery rate *q* values for each variable. Significant *P* and *q* values are given in bold.

A)

Immunoassay		Genome-wide het	Immune genes het	Body mass (g)	Sex
Lysozyme	β	0.552	-0.054	< 0.001	-0.120
n = 127	SE	0.206	0.090	< 0.001	0.032
	P	**0.009**	0.552	0.270	**< 0.001***
Haemolysis	β	-1.455	0.235	0.002	-0.157
n = 129	SE	0.935	0.338	0.001	0.122
	P	0.123	0.488	**0.029**	0.202
Bacteria-killing	β	-0.562	-0.052	< 0.001	-0.019
n = 128	SE	0.303	0.109	< 0.001	0.039
	P	0.066	0.632	0.649	0.636
Agglutination	β	1.261	-0.301	0.001	0.124
n = 118	SE	1.003	0.376	0.001	0.138
	P	0.212	0.425	0.335	0.373

*indicates that males have stronger lysozyme activity

B)

Immunoassay		Genome-wide het	MHCI	Blec1	TLR1B	TLR4	TLR5	TLR15	IL4	BD11	Body mass	Sex
Lysozyme	β	0.492	0.057	0.014	0.020	-0.043	-0.073	-0.013	0.034	-0.132	< 0.001	-0.141
$n = 114$	SE	0.240	0.051	0.043	0.038	0.078	0.080	0.040	0.039	0.104	< 0.001	0.038
	P	**0.045**	0.270	0.756	0.609	0.584	0.365	0.753	0.382	0.210	0.220	**< 0.001**
	q	0.245	0.594	0.756	0.745	0.745	0.601	0.756	0.601	0.594	0.594	**0.004***
Haemolysis	β	-0.880	0.160	-0.001	-0.083	-0.321	0.065	0.018	-0.082	0.005	0.003	0.035
$n = 113$	SE	0.698	0.117	0.113	0.095	0.201	0.201	0.101	0.097	0.236	0.001	0.099
	P	0.211	0.178	0.994	0.384	0.113	0.748	0.862	0.400	0.983	**0.001**	0.727
	q	0.580	0.580	0.994	0.733	0.580	0.994	0.994	0.733	0.994	**0.010**	0.994
Bacteria-killing	β	-0.429	0.056	-0.090	-0.019	-0.066	0.007	0.009	0.038	-0.081	< 0.001	-0.022
$n = 115$	SE	0.323	0.050	0.050	0.042	0.090	0.088	0.045	0.043	0.104	< 0.001	0.043
	P	0.187	0.265	0.078	0.659	0.465	0.938	0.845	0.380	0.435	0.387	0.621
	q	0.730	0.730	0.730	0.805	0.730	0.938	0.930	0.730	0.730	0.730	0.805
Agglutination	β	2.221	-0.086	-0.144	-0.004	-0.139	0.524	0.030	0.237	-0.374	0.002	0.129
$n = 105$	SE	1.126	0.170	0.175	0.144	0.326	0.307	0.153	0.144	0.343	0.001	0.148
	P	0.052	0.614	0.414	0.980	0.670	0.092	0.844	0.105	0.279	0.114	0.385
	q	0.285	0.744	0.592	0.891	0.744	0.285	0.844	0.285	0.558	0.285	0.592

*indicates that males have stronger lysozyme activity

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Publications
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Bateson, Z.W., L.A. Whittingham, J.A. Johnson, P.O. Dunn. 2015. Drift and selection shape MHC variation in prairie-chickens. Society for Integrative and Comparative Biology Conference, West Palm Beach, FL
Bateson, Z.W., P.O. Dunn, S.D. Hull, A.E. Henschen, J.A. Johnson, and L.A. Whittingham. 2014. The complexity of restoring genetic diversity in a threatened population of greater prairie-chickens. Midwest Bird Conservation and Monitoring Workshop. Port Washington, WI

Bateson, Z.W., P.O. Dunn, S.D. Hull, A.E. Henschen, J.A. Johnson, and L.A. Whittingham. 2013. Genetic analysis of a translocation of greater prairie-chickens into Wisconsin. The Wildlife Society 20th Annual Conference, Milwaukee, WI

Bateson, Z.W., P.O. Dunn, S.D. Hull, A.E. Henschen, J.A. Johnson, and L.A. Whittingham. 2013. Genetic restoration of a threatened population of greater prairie-chickens in Wisconsin. American Ornithologists' Union and Cooper Ornithological Society Conference, Chicago, IL.

Bateson, Z.W., P.O. Dunn, S.D. Hull, A.E. Henschen, J.A. Johnson, and L.A. Whittingham. 2013. Genetic analysis of a translocation of greater prairie-chickens into Wisconsin. Biological Sciences Research Symposium UW-Milwaukee, Milwaukee, WI

Bateson, Z.W. 2010. Living on the edge: Genetic diversity in peripheral populations of the common five-lined skink. MSU-Mankato Graduate Research Conference.

Bateson, Z.W. 2009. Sex may not mean total success: Paternity analysis of the common five-lined skinks (*Plestiodon fasciatus*). MSU-Mankato Graduate Research Conference.

Bateson, Z.W. 2008. Sperm precedence in squamates: May the best man win. Midwest Ecology and Evolution 28th Annual Conference, Athens, OH.

Public Presentations

Bateson, Z.W., P.O. Dunn, S.D. Hull, A.E. Henschen, J.A. Johnson, and L.A. Whittingham. 2015. Genetic restoration and monitoring of greater prairie-chickens in Wisconsin. Riveredge Nature Center's Student Research Symposium: Connections with Nature, Newburg, WI.

Bateson, Z.W., P.O. Dunn, S.D. Hull, A.E. Henschen, J.A. Johnson, and L.A. Whittingham. 2013. Genetic restoration of a threatened population of greater prairie-chickens. Riveredge Nature Center's Student Research Symposium: Connections with Nature, Newburg, WI.

Undergraduate mentoring project

Hack, D., J. Gurter, D.P. Toma, and **Z.W. Bateson**. 2010. Isolation of phytochelatin and metallothionin cDNA from the cattails *Typha latifolia* and *T. angustafolia*. MSU-Mankato Undergraduate Conference.

Invited Lectures

History and conservation of greater prairie-chickens in Wisconsin. Evolution and Ecology of Birds. April 29, 2015

Primer to population genetics. Introductory Genetics. April 22, 2011.

Research Grants

Doctoral Dissertation Improvement Grant, NSF (2015), $18,508
James D. Anthony Award, UW-Milwaukee (2015), $500
Ivy Balsam-Milwaukee Audubon Society Award (2015), $500
Joseph B. Baier Award, UW-Milwaukee (2014), $200
The American Ornithologists' Union Research Award (2013), $2460
Ruth Walker Grant-in-Aid, UW-Milwaukee (2012-15), $1500/year
Wisconsin Society for Ornithology Award (2013), $500

Sigma Xi Grants-in-Aid (2012), $500

Fellowships and Awards
Distinguished Dissertation Fellowship (2014-15), UW-Milwaukee
Chancellor's graduate award, UW-Milwaukee (2012-2015)
Best Graduate Student Talk (runner-up), Riveredge Nature Center Symposium (2015)
Best Graduate Student Talk, Biological Sciences Research Symposium UW-Milwaukee (2013)
Student Success Award, Student Success Center, MAP-Works UW-Milwaukee (2013)
Best Oral Presentation, Midwest Ecology and Evolution Conference Athens, Ohio (2008)

Teaching Experience
Teaching Assistant, Survey of Zoology & Foundations of Biological Sciences I, UW-Milwaukee (2011-present)
Adjunct Faculty, Genetics, MSU-Mankato (2010-11)
Teaching Assistant, Microbiology, Genetics, & General Biology, MSU-Mankato (2007-10)

Professional and Volunteer Services
Reviewer, Journal of Wildlife Management (2014-15)
Wisconsin Frog and Toad Call Survey (2012-15)
Wisconsin High School Science Fair Judge (2012-16)

Professional Memberships
Wisconsin Society for Ornithology
Society for the Study of Amphibians and Reptiles