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ORIGINAL PAPER

Parasite infection, but not immune response, influences paternity in western bluebirds

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Abstract Parasites can impose heavy costs on their hosts, and females may benefit from selecting mates that are parasite resistant and/or have a stronger immune response. Trade-offs between immune response and sexual signaling have been proposed as a mechanism to ensure signal honesty. Much of the work on sexual signaling and immune response does not consider parasites directly and thus cannot confirm whether a stronger immune response necessarily results in lower parasite burdens. Also, immunity is costly, and these costs can lower the overall fitness of individuals with too strong of an immune response. Males with immune responses of intermediate strength are therefore expected to be preferred by females and have the highest reproductive success. We tested whether immune response and blood parasite loads relate to sexual signaling and mating preferences in western bluebirds (*Sialia mexicana*). Immunity did not predict male reproductive success when considering either within- or extra-pair offspring, although a stronger immune response was correlated with lower parasite loads. However, uninfected males were more likely to sire extra-pair offspring than males infected with avian malaria. Thus, females were more likely to mate with uninfected males but not necessarily males with a stronger immune response. Our results may indicate that females select parasite-resistant males as mates to gain resistance genes for their offspring or that infected males are less likely to pursue extra-pair copulations.

Keywords Avian malaria · Extra-pair paternity · Immunity · Mate choice · *Sialia mexicana*

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Introduction

Sexual signals may have evolved to convey information to prospective mates about an individual's ability to resist parasites (Hamilton and Zuk 1982). Females selecting males with the most elaborate sexual signals may improve offspring fitness by gaining genes for parasite resistance, an idea known as the Hamilton–Zuk hypothesis (Hamilton and Zuk 1982). Numerous studies have tested this hypothesis by examining immune response in lieu of parasites (Møller et al. 1999), a logical step, since immunity constitutes one of the main lines of defense that organisms have against pathogens. However, immune responses are costly, and these costs may create trade-offs between immunity and other aspects of an organism's life history, such as reproduction (Sheldon and Verhulst 1996; Zuk and Stoehr 2002; French et al. 2009). Trade-offs between the immune system and reproductive effort have been invoked often in the context of sexual signaling, with sexual signals working as handicaps (Zahavi 1975, 1977; Getty 2002) so that only high-quality individuals can afford to pay the costs of producing a sexual signal while maintaining an appropriate immune response (Folstad and Karter 1992; Sheldon and Verhulst 1996), thus ensuring that sexual signals honestly reflect immunity and parasite resistance.

When examining how immunity relates to sexual signaling, one critical assumption is that stronger immunity results

in lower parasite burdens and greater parasite resistance. However, few studies test the validity of this assumption. Immunity may or may not correspond to parasite levels, depending upon the parasites involved and the method(s) with which one measures immunity (Adamo 2004). Thus, it is critically important to also examine parasite levels and how these relate to immune response and fitness (Graham et al. 2011), as higher measures of immunity may not always result in better parasite resistance and higher survival/fitness (Adamo 2004; Graham et al. 2011).

Studies examining the relationship between immunity and sexual signals often find conflicting results, with some reporting that individuals with a stronger immune response have better sexual signals, while others report the opposite pattern (Jacobs and Zuk 2012). One possible explanation for these discrepancies is that immunity and sexual signal quality may exhibit a non-linear relationship. Ecoimmunology studies that examine sexual signaling often implicitly assume that a stronger immune response is better for an individual. However, the costs of immunity may create a situation in which individuals benefit most by having intermediate levels of immunity (Viney et al. 2005). Too weak an immune response would result in the individual succumbing to pathogens, but too strong an immune response would be costly, both in terms of energy (Sheldon and Verhulst 1996; Schmid-Hempel 2003) and potential damage to the host's own tissues (Graham et al. 2005). These costs can have serious consequences; for example, breeding female eiders (*Somateria mollissima*) that mounted an immune response to a novel antigen had lower survival rates (Hanssen et al. 2004). Given that the birds were not infected with actual parasites, the reduced survival must have come from the costs of the immune response itself, indicating that a strong immune response can have negative fitness consequences for the host.

Work on wild birds supports the idea that an intermediate level of immunity results in higher fitness. Stjernman et al. (2008) found that blue tits (*Cyanistes caeruleus*) infected with intermediate levels of the blood parasite *Haemoproteus* had higher survivorship than individuals with very high or very low levels of infection. This implies that individuals that mount an immune response strong enough to drive parasite levels very low pay a significant cost. Similarly, other studies on blue tits (Råberg and Stjernman 2003) and kestrels (*Falco tinnunculus*; Kim et al. 2013) have found evidence of stabilizing, rather than directional, selection on immune response, implying that there is some intermediate, optimal level of immunity. If females select the fittest mates for good genes, they may benefit from selecting males with an intermediate level of immune response. Past work indicates that such males should have the highest survival probability, even if they do not have the lowest parasite load (Råberg and Stjernman 2003; Stjernman et al. 2008). Females selecting such males might gain indirect benefits from producing fitter offspring.

We set out to test whether immunity relates to parasite loads in the wild and whether having an optimal level of immune response predicted male reproductive success. Here, we examine parasite load, immune response, sexual signaling, and reproductive success in the western bluebird (*Sialia mexicana*). To determine parasite infection, we focus on Haemosporidian blood parasites such as *Plasmodium* spp. and closely related *Haemoproteus* spp. (hereafter referred to as avian malaria; Pérez-Tris et al. 2005). Haemosporidian parasites cause chronic, long-term infections in many of the birds they parasitize, and such chronic infections are ideal for testing predictions of the Hamilton–Zuk hypothesis (Hamilton and Zuk 1982). Moreover, such parasites are common in wild birds, and infection with these parasites can result in lower fitness (Atkinson and Van Riper 1991; Merino et al. 2000; Asghar et al. 2011).

Western bluebirds, like many passerines, are socially monogamous (Dickinson 2001; Griffith et al. 2002). Males will socially pair with a single female to raise the chicks but will also engage in extra-pair copulations with females at neighboring territories. Rates of extra-pair paternity are fairly high, with 27–45 % of nests containing extra-pair young, depending upon the population (Dickinson 2003; Duckworth 2006). Extra-pair paternity can increase overall male reproductive success (O'Brien and Dawson 2011) and also increases the variance of male reproductive success and the strength of sexual selection on males (Albrecht et al. 2009; Balenger et al. 2009a).

Plumage coloration acts as a sexual signal in closely related species, such as the mountain bluebird (*Sialia currocoides*; Balenger et al. 2009b) and may act as a sexual signal in western bluebirds as well. If an immune response of intermediate strength does confer the highest fitness, we expect females to prefer males that have such a response. Therefore, we predicted that coloration would show a non-linear relationship with immune response. We also predicted that individuals with a stronger immune response would have lower parasite burdens, and that males with an intermediate immune response would be preferred as mates and sire the most extra-pair offspring.

Materials and methods

Study species and field methods

Western bluebirds are medium-sized (23–30 g) passerines that nest in secondary cavities and readily inhabit human-made nestboxes (Guinan et al. 2008). In our population, they breed from May until August, and most pairs produce a single clutch of four to five eggs per season (range, two to seven eggs per clutch). Double brooding occurs rarely, with only around 5 % of pairs producing second clutches (Jacobs et al. 2013). Both

male and female western bluebirds display ultraviolet (UV)-blue coloration on the back, head, wings, and tail and a patch of chestnut coloration on the breast. Males, however, display brighter plumage than females (Jacobs 2013).

All field work was conducted at the Los Alamos National Laboratory (LANL) in Los Alamos, New Mexico. Los Alamos is located on the Pajarito Plateau at approximately 2200 m (7300 ft) in the Jemez Mountains in northern New Mexico. A network of nestboxes was established around the Plateau in 1997 and has been monitored every breeding season since then. This network consists of over 400 wooden nestboxes mounted on trees in ponderosa pine forests or piñon-juniper woodlands located in 37 sites (Fair and Myers 2002a; Jacobs et al. 2013). Boxes are mounted approximately 1–2 m above the ground, primarily on ponderosa pines (*Pinus ponderosa*) or piñon pines (*Pinus edulis*). Western bluebirds are the most common occupants of these boxes. During the course of this study (2010–2012), a large wildfire occurred in the area. In late June 2011, the Las Conchas fire burned over 150,000 acres in the Jemez Mountains (New Mexico Incident Information System 2011). Although this fire did not burn any of our field sites, it forced an evacuation of the area and disrupted field work for several weeks. For further details on field sites and box construction, see Fair et al. (2003) and Jacobs et al. (2013).

All parasite and coloration data were collected during the breeding seasons in 2010 through 2012. Immune measurements were carried out in the 2011 and 2012 seasons. We monitored nestboxes and recorded all active nests or nests in which we found eggs. For each box, we determined the species nesting there, the clutch size, the number of eggs that hatched, the hatching date, and the number of offspring fledged. In many cases, we did not record the date of clutch initiation because we found nests after the clutch had been completed. However, we use the Julian hatching date of the nestlings as our measure of the timing of breeding.

We captured adult birds at their boxes using either box traps or mist nets. This method primarily captures individuals coming to the box to feed nestlings (ACJ, personal observation), and we presumed that adults captured in front of a given box were the social parents of any nestlings inside that box. Upon capture, we fitted each individual with a US Fish and Wildlife Service numbered aluminum leg band and we measured the wing chord (±1 mm), tarsus length (±0.1 mm), and mass (±0.1 g) for each individual and took feather samples from the rump and chest regions for coloration analysis (see below). To determine age, we used the presence or absence of molt limits to classify individuals as second-year (SY) or after second-year (ASY) birds (Shizuka and Dickinson 2005). In cases where we could not determine age reliably (e.g., dull females, molting had already begun, etc.), we classified birds as after hatch-year (AHY). We also used a sterile needle to collect a blood sample of roughly 100 μL from the brachial vein. We placed a drop of blood on a slide and made a blood film, placed around 10–50 μL on an FTA® (Whatman Ltd.) card for DNA extraction and took the rest back to the lab for use in the bacteria-killing (BKA) assay (see below).

We captured some birds twice—the first time we inoculate birds with sheep red blood cells (SRBC; see below). We also collected feather and DNA samples from adults during this capture event. A week later, we recaptured birds to take blood for immune assays (see below). For these birds, we had three total measures of immune response: bacteria-killing ability (BKA) of the plasma, antibody response to SRBC, and leukocyte profiles (see below). For all other adults in the study, our immune measurements consisted of leukocyte profiles and BKA response. In some cases, we failed to recapture focal individuals injected with SRBC at the end of the second week; we had DNA and feather samples for these birds but no immune data (see below). Many of these individuals were birds we captured during the summer of 2011, when the Las Conchas wildfire disrupted our sampling.

When nestlings reached 9 days of age, we banded them and took a small (10–50 μL) blood sample from them for use in paternity analyses. We continued to monitor nests until all nestlings had fledged, at age 16 days or older (Fair and Myers 2002b). If we found nests empty before nestlings would have reached fledging age, we considered them depredated. In some cases, individual nestlings disappeared before reaching fledging age; we counted them as depredated as well. In a few cases, we found banded nestlings dead after their siblings had fledged; we collected and recorded these but did not attempt to determine the cause of death. All work was done in accordance with the Guidelines to the Use of Wild Birds in Research (Fair et al. 2010) and with the approval of the Los Alamos National Laboratory Institutional Animal Care and Use Committee.

Bacteria-killing assay

To measure immune function, we used a BKA measuring the ability of the plasma to kill bacteria in vitro, primarily via natural antibodies and complement (Millet et al. 2007; Liebl and Martin 2009). We used *Escherichia coli* (ATCC no. 8739) supplied in pellets containing 1×10^6 to 1×10^7 microorganisms per pellet (Epower Microorganisms, MicroBioLogics, St. Cloud, MN). Each pellet was reconstituted in 40 mL of sterile 1× phosphate-buffered saline (PBS) at 37 °C. This stock solution was then diluted down to make a working solution of 2×10^5 microorganisms/mL. We made a fresh stock solution every week and kept all bacterial solutions at 4 °C at all times. We followed the procedures from Liebl and Martin (2009). Briefly, we mixed plasma with 1× PBS and the bacterial solution (approximately 2.5×10^6 CFU per sample). This mixture was then incubated for 30 min at 37 °C. After the initial incubation, we added 250 μL of sterile tryptic soy broth.

We also prepared a sterile blank and a positive control. All samples were then incubated for 12 h at 37 °C. We used a Tecan Infinite 200 (Tecan Group Ltd., Männedorf, Switzerland) to measure the absorbance of the samples at 300 nm. We calculated the antimicrobial activity of the plasma as 1- (absorbance of sample/absorbance of positive control). We ran samples in triplicate when we had sufficient plasma volumes and in duplicate when we did not, and we averaged the results from replicates to get a single value for each individual.

Antibody response

To measure the ability of an individual to mount an antibody response to a novel antigen, we challenged birds using SRBC. We captured adults at a select set of nests shortly after the nestlings had hatched. Captured birds were banded to ensure individual identification and then injected intraperitoneally with 0.075 mL of a 10 % SRBC solution in 10× PBS (Sigma-Aldrich Company, St. Louis, MO). Basic measurements, feather samples, and a 10–50-μL blood sample for DNA were also taken at this time. Birds were then released, and 1 week following the initial injection, we recaptured the birds and collected a 20–50-μL sample of blood in an unheparinized microhematocrit tube. We also collected blood for the bactericide assay and slides at this time. Blood samples were taken from the wing vein using sterile methods. We successfully recaptured and collected blood from 18 individuals (ten females and eight males).

We spun the blood down and froze the resulting serum sample at −70 °C until we could perform the assay. Before beginning the assay, we first heated the serum samples to 56 °C for 30 min in a hot water bath. We then serially diluted serum samples and added SRBC to each well, then recorded the highest dilution in which we observed agglutination (Fair et al. 2003).

Leukocyte and parasite counts

A single drop of blood was placed on a slide and spread to form a blood film. Once the blood had air dried, we fixed the slide in 100 % methanol and stained it using Wright–Geimsa stain (Dein 1984). We examined all blood films under ×1000 magnification. For each slide, we conducted two counts of 100 leukocytes, classifying these as lymphocytes, heterphils, monocytes, eosinophils, or basophils. We then averaged the results of both counts and took the ratio of heterophils/lymphocytes (H/L ratio). We did not use total leukocyte count as a measure of immunity, as this can be difficult to interpret (Salvante 2006).

Each slide was examined for a minimum of a half hour to look for parasites. If we detected an infection, we counted a minimum of 5000 red blood cells (RBCs) and quantified the proportion of these that contained parasites (parasitemia). One person (ACJ) conducted all cell and parasite counts to eliminate variation between observers. We could not readily distinguish between *Haemoproteus* and *Plasmodium* infections in the slides, but subsequent molecular analyses revealed that both genera were present (see below). Aside from malaria infections, the only other parasite detected in the blood films was an infection by an unidentified filarial nematode in one individual; we did not include this in the analysis.

Molecular analyses: detection of malaria

We eluted DNA from the FTA cards according to the manufacturer's instructions and confirmed that all samples contained 10–150 ng/μL of DNA using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE). To determine whether birds were infected with avian malaria, we used a nested polymerase chain reaction (PCR) reaction following the methods described in Waldenström et al. (2004). In cases where the PCR gave us different results from the slides (e.g., if the PCR indicated that the bird was infected but no parasites were detected on the slide), we ran the reaction twice to confirm our results. If the second reaction confirmed the presence of the parasite, we counted the bird as infected.

We took products from the nested PCR reaction from five birds and sequenced them to determine the strains of parasite infecting the population. Once we had obtained sequences, we used the Basic Local Alignment Search Tool (BLAST) to locate similar sequences. We determined that the parasites fell into two general categories: some birds were infected with a strain of *Plasmodium*, while others were infected with a strain of *Haemoproteus*. This corresponds with other studies looking at avian malaria in western bluebirds (Ricklefs and Fallon 2002; Martinsen et al. 2008). MalAvi, the database for information on avian haemosporidian parasites (Bensch et al. 2009), indicates that two strains of avian malaria have been detected in this species. One strain belongs to the genus *Haemoproteus* and also infects the closely related eastern bluebird (*Sialia sialis*; Ricklefs and Fallon 2002). The other strain is classified as a variety of *Plasmodium relictum* (Martinsen et al. 2008).

To quantify the intensity of individual infections, we used a quantitative PCR (qPCR) reaction. We repeatedly measured initial DNA concentration, and then diluted all samples down to 5 ng/μL. We used our sequence data to develop a primer pair: HaemSmex For (5′-CCTTGGGGTCAAATGAGTTT-3′) and HaemSmex Rev (5′-TCCACCACAAATCCATGAAA-3′). These primers were designed to amplify a section of mitochondrial DNA in the parasites from both the *Haemoproteus* lineage and the *Plasmodium* lineage. We were unable to design working lineage-specific primers to distinguish between these two genera.

We carried out qPCR reactions using a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Each reaction was 25 μL in volume and included 2 μL of template DNA (5 ng/μL), 0.5 μL of forward and reverse primer, 12.5 μL SYBR Green Supermix, and 10 μL ddH_2O. Thermocycling conditions were as follows: after an initial incubation of 95 °C for 3 min, we ran 40 thermal cycles (95 °C for 10 s, 55 °C for 10 s, and 72 °C for 30 s). We ran each sample in triplicate and excluded obvious outliers from the analysis. To generate a standard curve, we used DNA from a bird we knew to be heavily infected from slide data. We used serial dilutions of this sample to generate a standard curve which we used to estimate the parasitemia of unknown samples. All reactions used had an efficiency of between 90 and 115 %. In most experiments, the Ct value (the number of cycles needed to reach the detection threshold) ranged between 24 and 35 cycles. We visually inspected all melt curves and rejected samples with abnormal curves that indicated the presence of primer dimers.

Paternity analyses

To assign paternity, we used highly variable microsatellite loci previously described for bluebirds. We used the loci Smex5, Smex9, and Smex14 (Ferree et al. 2008) and Sialia36 and Sialia37 (Faircloth et al. 2006). PCR products were sized using an ABI 3100 Genetic Analyzer, and we confirmed product size visually using GeneMapper v4.1. To assign extra-pair offspring to their genetic fathers, we used CERVUS 3.0 (Kalinowski et al. 2007). None of the allele frequencies deviated from Hardy–Weinberg equilibrium, and the combined probability of non-exclusion was 0.008 when the mother was known. In cases where offspring showed no mismatches in alleles relative to the social father, we classified them as within-pair offspring. We assigned offspring to extra-pair sires if CERVUS assigned paternity to a given male with 95 % confidence or with 80 % confidence if the putative father was from the same field site or a field site in close proximity to the nest. We used this criterion because most extra-pair males come from adjacent territories (Dickinson 2001; Akçay et al. 2012).

Coloration analyses

We collected nine feathers from each region on the body we measured. For this study, we focus on UV-blue feathers collected from the rump, which exhibit UV-blue, structural-based coloration (Siefferman and Hill 2003). We chose this body region because UV-blue coloration plays a role in social mate choice in this species (Jacobs 2013). We taped feathers to a black, non-reflective background in an overlapping fashion to mimic how they would lie on the birds (Siefferman and Hill 2003). We then measured reflectance relative to a white standard using an Ocean Optics USB4000 spectrophotometer (range, 200–1100 nm; Ocean Optics Inc., Dunedin, Florida, USA) with a xenon light source. We took five readings by lifting up and replacing the probe; these readings were subsequently averaged. Using these readings, we calculated repeatability for hue, chroma, and brightness (see below) according to Lessells and Boag (1987). All three showed significant variation among rather than within individuals and high repeatability (hue: $P<0.001$, $R=0.856$; chroma: $P<0.001$, $R=0.839$; brightness: $P<0.001$, $R=0.781$).

We used three measures to describe individual coloration: hue, chroma, and brightness. To measure brightness, we took the average reflectance value across the 300–700-nm spectrum. To measure chroma, we summed the reflectance values from 300 to 500 nm for the (UV-blue region) and divided that by the total summed reflectance over the whole spectrum. We calculated the hue as the wavelength of peak reflectance.

Statistical analyses

We performed analyses in JMP 11 (SAS Institute 2014), with the exception of the analyses of parasitemia data (see below). Unless otherwise stated, all response variables had a normal distribution of errors. In some cases, we captured the same individual in multiple years; however, in most cases, we were missing measurements for that individual in one or more years (e.g., no immune response data collected in 2010, sampling interrupted by the fire in 2011). Thus, rather than using a repeated measure analysis, we included each individual in the analysis only once to avoid pseudoreplication. When selecting which points to include, we chose to include the data for the year from which we had collected the most information (e.g., all immune assay data). In the few cases where we had complete data on the same individual for both years, we randomly selected 1 year to include. Due to the small sample sizes for the SRBC antibody assay, we analyzed these data separately to avoid reducing sample sizes for all analyses. SRBC data were log transformed before analysis. We used a *t* test to compare the antibody response of infected versus uninfected individuals.

Parasitimia data from both slides and qPCR were non-normally distributed, with a few of the hosts harboring high parasite loads while most carried only light infections or no infection at all. Transformation of the data did not improve the distribution. To examine which factors affected individual parasitemia, we used mixed effects models with a zero-inflated negative binomial distribution (using the "glmmADMB" function in the "glmmADMB" package in R). This model takes into account the high number of zeros in the response variable due to uninfected individuals. We used parasitemia as our response variable and mass, sex, breed date, and immune response (BKA) as predictors. Year was

included as a random factor. In uninfected birds, we could not distinguish between birds that had successfully fought off an infection versus those that had never been exposed. Therefore, we also used a Spearman rank correlation to determine whether immune response was correlated with parasitemia in birds that were already infected. In some cases, we had DNA samples but no slide data for infected individuals. We identified eight individuals as infected using molecular methods, but we were unable to detect parasites using the slides. Unless otherwise stated, all data on infection intensity/parasitemia are taken from the qPCR results.

When examining coloration, we chose to focus on male UV-blue brightness, as measured by the overall brightness of the rump feathers. This measure is variable between individuals and plays a role in assortative mating and male paternity (Jacobs 2013). We ran a series of general linear models to determine the effects of immune response (BKA), mass, infection status, age, and H/L ratio on male coloration, within-pair paternity, and overall reproductive success (including all surviving within-pair and extra-pair young sired by that male). We included year as a random factor initially, but this factor was not significant and did not alter the results. Removing year allowed us to run a forward stepwise regression using minimum AIC_c as the criteria. We ran each model with BKA to test for a positive, linear effect of immune response and then again with BKA squared instead of BKA to test for non-linear effects of immune response and compared the two using AIC_c scores. We used a logistic regression to test whether infection status (infected versus not), age, mass, breeding date, or immune response (BKA) affected a male's probability of siring extra-pair young and of losing paternity in his own nest.

Results

Parasite infection

We were able to obtain parasite data using molecular methods for 119 individual birds, some of which we caught in multiple years. Of these, 48 (40.3 %) were infected with avian malaria, 23 females and 25 males. In three cases, recaptured individuals that had been infected showed no infection when caught the next year, indicating a clearance of the infection. We used the data from the year in which these individuals had been infected. Infected individuals did not differ from uninfected individuals in terms of mass, BKA response, or H/L ratio ($F_{3, 63}$=0.33, P=0.80).

Parasitemia measured using qPCR was highly correlated with the parasitemia recorded using slides (r_s=0.70, n=46, P<0.001). When we tested for the effects of immune response on parasitemia across all individuals, immune response (BKA) was not a significant predictor (log-likelihood ratio test: χ^2=2.39, P=0.12). Mass, breeding date, and sex also had no significant effect on parasitemia (all P>0.20). However, when we focused on the parasitemia within infected individuals, individuals with a stronger BKA response had significantly lower parasitemia (r_s=−0.36, n=31, P=0.05; Fig. 1).

We found no differences between the sexes in terms of BKA or H/L ratio ($F_{2, 65}$=0.96, P=0.39). We found a marginally non-significant difference in antibody response to SRBC between infected and uninfected individuals (t_{14}= 1.97, P=0.07); uninfected individuals tended to have stronger responses. SRBC response did not correlate with parasitemia (r_s=−0.24, n=15, P=0.38), but this might be due to small sample sizes (15 infected individuals for whom we had both SRBC and parasitemia data).

Coloration and immune response

UV-blue brightness in males was unrelated to infection status, BKA, mass, or breed date. The best performing model selected by the stepwise procedure included only the intercept, and none of the factors was significant in the full model (all P>0.40). Using BKA squared in place of BKA did not alter the results, and the full model with BKA squared did not differ from the full model with BKA (full model with BKA: AIC_c= 236.02; full model with BKA squared: AIC_c=236.00). This indicates that there is no polynomial relationship between immune response (BKA) and coloration.

Male reproductive success, immune response, and parasites

We genotyped 324 nestlings over 3 years; 114 of which we classified definitively as extra-pair offspring (35.2 %). Of the 76 nests for which we genotyped nestlings, 51 of them (67 %) contained at least one extra-pair offspring. In many cases, we were unable to confidently assign paternity to nestlings. We

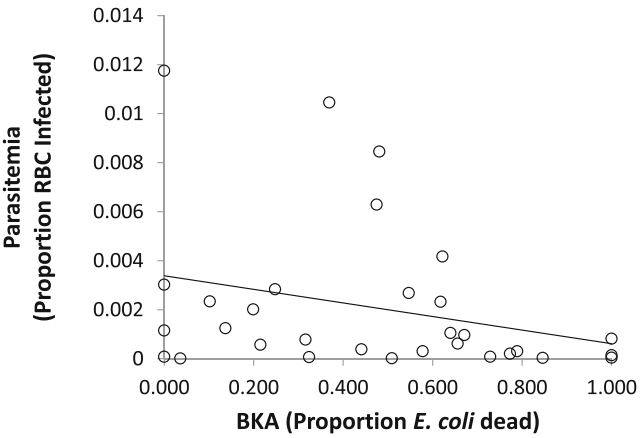

Fig. 1 The relationship between immune response (as measured by the bacteria-killing assay) and the proportion of RBCs infected with avian malaria, excluding uninfected individuals

assigned 31 extra-pair offspring to 19 different fathers. Six nestlings (five within-pair and one extra-pair) disappeared or died of unknown causes after we had collected DNA samples but before fledging. We included data from these nestlings in calculating the proportion of within-pair young in a given nest but did not include them when determining a male's overall reproductive success for a given year.

We found no evidence that the likelihood of a male losing paternity in his own nest was affected by male age, mass, breeding date, immune response (BKA), or infection with avian malaria ($\chi^2_{6,\ 35}$=3.86, P=0.70). However, we did find an effect of infection on the probability that a male would sire extra-pair offspring in another nest ($\chi^2_{6,\ 33}$=13.95, P=0.03). Males that were more likely to sire extra-pair offspring tended to be uninfected (Wald $\chi^2_{1,\ 33}$=4.91, P=0.03; Fig. 2) and heavier (Wald $\chi^2_{1,\ 33}$=4.44, P=0.04; Table 1). Males that sired extra-pair young tended to be older, but this trend did not reach significance (Wald $\chi^2_{1,\ 33}$=3.36, P=0.07; Table 1). We tested whether mass, breed date, year, infection, or immune response resulted in differences in the proportion of within-pair young in a male's nest but found no effect of any of these factors (full model: all P>0.30). The stepwise procedure indicated that the best model included only the intercept. Similarly, mass, breed date, infection, and immune response all failed to explain differences in total male reproductive success (full model: all P>0.10); the stepwise procedure failed to include any of these factors in the best model. Running the models with BKA squared, to test for non-linear effects of immune response, produced the same results.

Discussion

Male bluebirds infected with avian malaria were less likely to sire extra-pair offspring. One explanation for this is that females may prefer unparasitized males as extra-pair partners.

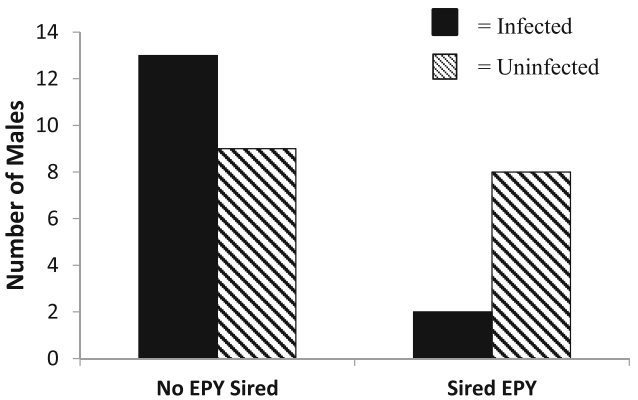

Fig. 2 The number of males who successfully sired extra-pair offspring versus those that did not. *Solid bars* indicate individuals infected with malaria; *cross-hatched bars* indicate uninfected individuals

Table 1 Results of the logistic regression performed to determine whether individual traits affected the probability that a male would sire extra-pair offspring

Variable	β	Wald Chi-square	*P* value
Mass	−0.86	4.44	0.035
Breed date	0.01	0.05	0.818
Infection (0 versus 1)	−1.60	4.91	0.027
BKA	−0.67	0.15	0.700
Age (AHY versus SY)	0.47	0.27	0.601
Age (ASY versus SY)	−1.33	3.36	0.067

We considered mass, breeding date (as indicated by the hatch date of the nestlings), infection status (0=uninfected, 1=infected), immune response (bactericidal capacity of the blood/BKA), and age as predictor variables

Females did not, however, prefer unparasitized males as social mates, perhaps because social mate choice is constrained by other factors, such as nest site availability. Bluebirds are secondary cavity nesters, and nest sites may be limited in the wild (Guinan et al. 2008). The presence of nestboxes may help alleviate this pressure; of the more than 400 nestboxes available across the study area, only approximately 120 were occupied each year by all species. However, in certain sites, occupancy rates in boxes could be much higher, with 100 % occupancy at one site. Thus, nest site availability may still constrain female choice in this system, with females potentially accepting subpar social mates to gain access to a nest site. Females may also seek other characteristics in social mates that are indicative of direct benefits such a good territory or a high level of paternal care. Since females gain no direct benefits from extra-pair mates, they could engage in extra-pair matings to obtain good genes for their offspring (Griffith et al. 2002; but see Akçay and Roughgarden 2007).

Another possible explanation for this finding is that infected males may be less likely to seek out extra-pair copulations. Female western bluebirds exert active control over extra-pair copulations (Dickinson 2001), but they do not seem to actively seek out extra-pair males, unlike other species of passerines (Chiver et al. 2008). Rather, males from neighboring territories foray into the female's territory and solicit copulations (Dickinson et al. 2000; Dickinson 2001). Thus, this pattern could also arise if infected males are less likely to make forays into their neighbors' territories, possibly because of changes in male aggressive behavior due to parasites. Studies on male red grouse (*Lagopus lagopus scoticus*) treated with antihelmintic drugs to clear their nematode infections found that treated males displayed more aggressive behaviors and were more territorial than infected males (Fox and Hudson 2001; Mougeot et al. 2005). Aggression can be related to testosterone levels, and higher testosterone can result in males siring more extra-pair young (Raouf et al. 1997). However, Duckworth (2006) found that more aggressive male bluebirds

did not have greater success siring extra-pair offspring. Aggressive behavior and territory defense could also include better ability to exclude extra-pair males from the territory, and better mate guarding overall. However, we found no indication that uninfected males were less likely to lose paternity in their own nests than their infected counterparts.

If female choice determines which males sire extra-pair young, females may be selecting extra-pair mates that may carry genes for parasite resistance (Hamilton and Zuk 1982). Females may also prefer uninfected mates to avoid becoming infected themselves (Clayton 1991; Able 1996), but this is unlikely to apply to the spread of avian malaria, which requires a vector, usually a biting fly, in which the parasite must develop before becoming infectious (Atkinson and van Riper 1991). Females are not the ones traveling to other territories to seek extra-pair copulations in this species; thus, it seems unlikely that they would be exposed more often to these infectious mosquitoes simply because they mate with a parasitized male. Given the transmission route of the parasite, engaging in extra-pair copulations with an infected individual likely does not increase the risk of infection.

The strength of the immune response did not predict male sexual signaling or reproductive success, despite being related to lower parasite burdens. Possibly the relationship between immune response and lower parasite burdens is too weak for any effect of immunity to be detected in our study. We may also not have captured a full picture of the birds' immune response with the measures that we used. While bacteria-killing ability and antibody response to SRBC are both common ways of measuring immunity, they are hardly the only methods, and each method captures a slightly different aspect of the immune response (Salvante 2006). We cannot rule out the possibility that, had we measured other branches of the immune system, we might have found a connection.

While trade-offs between immunity and reproductive effort are predicted by life history, such trade-offs may only be apparent when individuals face poor environmental conditions, such as a lack of food (French et al. 2009). This could explain why we detected no relationship between plumage coloration and immunity. However, during 2 of the 3 years of study (2011 and 2012), a severe drought hit the study area, and many nestlings died of unexplained causes, possibly starvation or disease. These years were also when we collected our data on immune response. Droughts cause many ill effects for bluebird nestlings (Fair and Whitaker 2008) and presumably cause stress in the adults as well. Thus, the majority of individuals used in our study would have experienced poor conditions and we should have detected a trade-off between immunity and reproduction.

We found no relationship between immune response and parasite load when we considered all individuals (both infected and uninfected). However, many individuals that were uninfected may never have been exposed to the disease. Our results indicate that it is possible for birds to clear an infection; however, this was a rare occurrence and sometimes occurred after the individual had been infected for multiple years. Thus, we assume that most of the birds in which we did not detect an infection had probably never been infected. When we considered infected individuals only, we did find that stronger immunity corresponded to lower parasite loads. However, it is also possible that a strong immune response may prevent an infection from ever establishing; without controlled infection studies, we cannot assess this possibility. We also have insufficient data to assess the role of immunity in clearing an infection.

Our finding that individuals with a strong BKA response had fewer parasites may have resulted from a stronger immune response suppressing the infection. While BKA measures the killing of bacteria, it has been correlated with non-bacterial parasites, including ectoparasites (Girard et al. 2011) and viral diseases (Wilcoxen et al. 2010). There is indirect evidence that the bacteria-killing ability of the blood can help birds fight infection in the wild; Florida scrub-jays (*Aphelocoma coerulescens*) with a stronger BKA response were more likely to survive an epidemic of eastern equine encephalitis virus (Wilcoxen et al. 2010). However, we cannot say for certain whether the trend we observed resulted from the activity of complement and natural antibodies, which are measured by the BKA response (Millet et al. 2007), or whether BKA correlates with some other mechanism of immunity which acts against the malaria parasites. Various components of the immune system may interact and trade off with one another (Forsman et al. 2008; Palacios et al. 2012), and it is possible that a strong response in BKA correlates with a strong response in a different branch of the immune system that may help to fight off malaria. Also, the relationship we observed may be due to parasites altering the immune system. Parasites can interact with the immune system in a variety of ways, including immune evasion and blocking of certain reactions within the immune system (Schmid-Hempel 2008). Thus, we may have detected differences in immune response because more heavily infected individuals have reduced immune defenses as a result of parasitism.

Interestingly, we did not find any influence of immune response or infection on male plumage coloration. This contradicts other studies, which have found that infection with parasites reduces the quality of sexual signals (Møller 1990, 1991; Zuk et al. 1990) or that individuals with better plumage-based sexual signals can better survive or cope with infection (Nolan et al. 1998; Lindström and Lundström 2000). However, in eastern bluebirds, coloration signals direct benefits such as male parental investment (Siefferman and Hill 2003, 2005) and could signal similar qualities in our species. Females would then have no reason to select brighter males as extra-pair partners, as they gain no direct benefits from such partners. The lack of association between plumage coloration

and infection implies that if females use male infection status as a criterion for mate choice, they must have some other way of assessing male health. It is unlikely that they use overall male condition, as we found no differences in mass between infected and uninfected individuals. Possibly, behavioral differences exist between males infected with malaria and uninfected males. High parasite loads can reduce other aspects of male sexual displays, such as song rate (Møller 1991).

We found no effects of immune response or parasite infection on overall male reproductive success. This is surprising, given that uninfected males tend to sire more extra-pair offspring. However, predation accounts for most of the nest failures in our population and other populations of western bluebirds (Kozma and Kroll 2010). Predation of nestlings is unlikely to depend on the infection status of the adults. Also, we may have underestimated male reproductive success if males sired extra-pair offspring in nests we did not sample. Some nests were depredated before the nestlings were old enough for us to obtain samples, and there may have been nests in nearby natural cavities whose nestlings we did not sample.

When birds become infected with avian malaria, they pass through two phases of the infection. During the first, acute phase, parasitemia levels spike, and many infected individuals die (Atkinson and van Riper 1991; Zehtindjiev et al. 2008; Asghar et al. 2012). This is followed by a much longer, chronic infection period, during which the parasites persist in the host at low, but steady, levels (Atkinson and van Riper 1991; Asghar et al. 2012) and have subtle, but still detrimental, effects on host fitness (Merino et al. 2000; Asghar et al. 2011). The birds in our study population were harboring chronic malaria infections, as indicated by low levels of parasitemia. These values may still provide us with some indication of how heavily infected individuals were during the acute phase (Asghar et al. 2012). However, detecting infection during the chronic phase can be difficult. In many cases, individuals with very low parasite counts may be categorized as uninfected when using slides, leading to an underestimation of the true parasite prevalence in the population. We encountered this problem in our study when using slide data. Of the 48 individuals that we identified as infected using molecular methods, there were eight (17 %) for which we did not detect any infection using slides. Also, because we only sampled breeding individuals, if heavily infected birds are less likely to hold territories and secure mates, we may have missed them in our sampling and underestimated the prevalence and intensity of infection.

Molecular methods have shown that birds from our population are infected with a strain of *Haemoproteus* (Ricklefs and Fallon 2002). Our sequence data confirm that this parasite is present, as well as a strain of *Plasmodium*. However, we were unable to distinguish between these species using slide data, and thus cannot say which birds were infected with *Plasmodium* and which were infected with *Haemoproteus*. Different lineages of avian malaria can impose different costs on their hosts. For example, Asghar et al. (2011) found that infection with a specific lineage of *Haemoproteus payevskyi* (lineage GRW1) was associated with later arrival of female great reed warblers (*Acrocephalus arundinaceus*) at the breeding site, while the other lineages investigated did not show this pattern. Thus further, detailed analysis of exactly which lineages infected which birds could prove fruitful.

In conclusion, we found evidence that uninfected males are more successful at siring extra-pair offspring, but immune response did not influence male paternity, despite being correlated with lower parasite burdens. This implies that parasite infection plays a larger role than immune response in sexual selection in this species. In future, to properly tease out the effects of parasites on mate choice and the relationships between parasite load and immunity, studies employing experimental infections would prove useful. Such manipulations would allow us to determine if individuals with stronger immunity can truly fight off infection better than those with a weaker immune response.

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Ethical standards This experiment was conducted in compliance with the current laws of the USA. Our research protocols were approved by the Animal Care and Use Committee at the Los Alamos National Laboratory (protocol no. 10-60).

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