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-pair 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 may 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*

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 Stoerh 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).

63 Studies examining the relationship between immunity and sexual signals often find
64 conflicting results, with some reporting that individuals with a stronger immune response have
65 better sexual signals, while others report the opposite pattern (Jacobs and Zuk 2012). One
66 possible explanation for these discrepancies is that immunity and sexual signal quality may
67 exhibit a non-linear relationship. Ecoimmunology studies that examine sexual signaling often
68 implicitly assume that a stronger immune response is better for an individual. However, the costs
69 of immunity may create a situation in which individuals benefit most by having intermediate
70 levels of immunity (Viney et al. 2005). Too weak an immune response would result in the
71 individual succumbing to pathogens, but too strong an immune response would be costly, both in
72 terms of energy (Sheldon and Verhulst 1996; Schmid-Hempel 2003) and potential damage to the
73 host's own tissues (Graham et al. 2005). These costs can have serious consequences; for
74 example, breeding female eiders (*Somateria mollissima*) that mounted an immune response to a
75 novel antigen had lower survival rates (Hanssen et al. 2004). Given that the birds were not
76 infected with actual parasites, the reduced survival must have come from the costs of the immune
77 response itself, indicating that a strong immune response can have negative fitness consequences
78 for the host.
79 Work on wild birds supports the idea that an intermediate level of immunity results in
80 higher fitness. Stjernman et al. (2008) found that blue tits (*Cyanistes caeruleus*) infected with
81 intermediate levels of the blood parasite *Haemoproteus* had higher survivorship than individuals
82 with very high or very low levels of infection. This implies that individuals that mount an
83 immune response strong enough to drive parasite levels very low pay a significant cost.
84 Similarly, other studies on blue tits (Råberg and Stjernman 2003) and kestrels (*Falco*
85 *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 don't 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 (*Siala 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 4 – 5 eggs per season (range 2 – 7 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 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 2,200 m (7,300 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 meters 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 pers. obs.), 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 U.S. 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

154 regions for coloration analysis (see below). To determine age, we used the presence or absence
155 of molt limits to classify individuals as second-year (SY) or after second-year (ASY) birds
156 (Shizuka and Dickinson 2005). In cases where we could not determine age reliably (e.g. dull
157 females, molting had already begun, etc.), we classified birds as after hatch-year (AHY). We also
158 used a sterile needle to collect a blood sample of roughly 100 µL from the brachial vein. We
159 placed a drop of blood on a slide and made a blood film, placed around 10-50 µL on an FTA®
160 (Whatman Ltd.) card for DNA extraction, and took the rest back to the lab for use in the bacteria-
161 killing (BKA) assay (see below).

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

172 When nestlings reached nine days of age, we banded them and took a small (10-50 µL)
173 blood sample from them for use in paternity analyses. We continued to monitor nests until all
174 nestlings had fledged, at age 16 days or older (Fair and Myers 2002b). If we found nests empty
175 before nestlings would have reached fledging age, we considered them depredated. In some
176 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 bacteria killing assay (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 #8739) supplied in pellets containing $1x10^6$ to $1x10^7$ microorganisms per pellet (Epower Microorganisms, MicroBioLogics, St. Cloud, MN). Each pellet was reconstituted in 40 mL of sterile 1X phosphate buffered saline (PBS) at 37°C. This stock solution was then diluted down to make a working solution of $2x10^5$ microorganisms per mL. We made a fresh stock solution every week and kept all bacterial solutions at 4ºC at all times. We followed the procedures from Liebl and Martin (2009). Briefly, we mixed plasma with 1X PBS and the bacterial solution (approximately $2.5x10^6$ CFU per sample). This mixture was then incubated for 30 minutes 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 hours 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 10x 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 one 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 (10 females and 8 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 minutes 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 1000x 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 5,000 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 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 qPCR reaction. We repeatedly measured initial DNA concentration, 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.

263 We carried out qPCR reactions using a CFX Connect Real-Time PCR Detection System
264 (Bio-Rad, Hercules, CA) with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Each
265 reaction was 25 µL in volume and included 2 µL of template DNA (5 ng/µL), 0.5 µL of forward
266 and reverse primer, 12.5 µL SYBR Green Supermix, and 10 µL ddH_2O. Thermocycling
267 conditions were as follows: after an initial incubation of 95°C for 3 minutes, we ran 40 thermal
268 cycles (95°C for 10 seconds, 55°C for 10 seconds, and 72°C for 30 seconds). We ran each
269 sample in triplicate and excluded obvious outliers from the analysis. To generate a standard
270 curve, we used DNA from a bird we knew to be heavily infected from slide data. We used serial
271 dilutions of this sample to generate a standard curve which we used to estimate the parasitemia
272 of unknown samples. All reactions used had an efficiency of between 90-115%. In most
273 experiments, the Ct-value (the number of cycles needed to reach the detection threshold) ranged
274 between 24 and 35 cycles. We visually inspected all melt curves and rejected samples with
275 abnormal curves that indicated the presence of primer-dimers.
276

277 *Paternity Analyses*

278 To assign paternity, we used highly variable microsatellite loci previously described for
279 bluebirds. We used the loci Smex5, Smex9, and Smex14 (Ferree et al. 2008) and Sialia36 and
280 Sialia37 (Faircloth et al. 2006). PCR products were sized using an ABI 3100 Genetic Analyzer,
281 and we confirmed product size visually using GeneMapper v4.1. To assign extra-pair offspring
282 to their genetic fathers, we used CERVUS 3.0 (Kalinowski et al. 2007). None of the allele
283 frequencies deviated from Hardy-Weinberg equilibrium, and the combined probability of non-
284 exclusion was 0.008 when the mother was known. In cases where offspring showed no
285 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 300-500 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 one 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 8 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^2 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 a 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: $\chi^2 = 2.39$, $P = 0.12$). 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, 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 ($r_s = -0.24$, $n = 15$, $P =$

374 0.38), but this might be due to small sample sizes (15 infected individuals for whom we had both
375 SRBC and parasitemia data).
376
377 *Coloration and Immune Response*
378 UV-blue brightness in males was unrelated to infection status, BKA, mass, or breed date.
379 The best performing model selected by the stepwise procedure included only the intercept, and
380 none of the factors was significant in the full model (all $P > 0.40$). Using BKA squared in place
381 of BKA did not alter the results, and the full model with BKA squared did not differ from the full
382 model with BKA (Full model with BKA: $AIC_c = 236.02$; full model with BKA squared: $AIC_c =$
383 236.00). This indicates that there is no polynomial relationship between immune response (BKA)
384 and coloration.
385
386 *Male Reproductive Success, Immune Response, and Parasites*
387 We genotyped 324 nestlings over three years, 114 of which we classified definitively as
388 extra-pair offspring (35.2%). Of the 76 nests for which we genotyped nestlings, 51 of them
389 (67%) contained at least one extra-pair offspring. In many cases, we were unable to confidently
390 assign paternity to nestlings. We assigned 31 extra-pair offspring to 19 different fathers. Six
391 nestlings (five within-pair and one extra-pair) disappeared or died of unknown causes after we
392 had collected DNA samples but before fledging. We included data from these nestlings in
393 calculating the proportion of within-pair young in a given nest, but did not include them when
394 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. 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. However, during two of the three 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 as 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; Lindstöm 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,

552 studies employing experimental infections would prove useful. Such manipulations would allow
553 us to determine if individuals with stronger immunity can truly fight off infection better than
554 those with a weaker immune response.

555

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568

Ethical Standards

This experiment was conducted in compliance with the current laws of the United State of America. Our research protocols were approved by the Animal Care and Use Committee at the Los Alamos National Laboratory (Protocol #10-60).

References

Able DJ (1996) The contagion indicator hypothesis for parasite-mediated sexual selection. P Natl Acad Sci USA 93:2229-2233

Adamo SA (2004) Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. J Insect Physiol 50:209-216

Akçay E, Roughgarden J (2007) Extra-pair paternity in birds: review of the genetic benefits. Evol Ecol Res 9:855-868

Akçay Ç, Searcy WA, Campbell E, Reed VA, Templeton CN, Hardwick KM, Beecher MD (2012) Who initiates extrapair mating in song sparrows? Behav Ecol 23:44-50

Albrecht T, Vinkler M, Schnitzer J, Poláková R, Munclinger P, Bryja J (2009) Extra-pair fertilizations contribute to selection on secondary male ornamentation in a socially monogamous passerine. J Evol Biol 22:2020-2030

Asghar M, Hasselquist D, Bensch S (2011) Are chronic avian haemosporidian infections costly in wild birds? J Avian Biol 42:530-537

Asghar M, Westerdahl H, Zehtindjiev P, Ilieva M, Hasselquist D, Bensch S (2012) Primary peak and chronic malaria infection levels are correlated in experimentally infected great reed warblers. Parasitology 139:1246-1252

Atkinson CT, van Riper C (1991) Pathogenicity and epizootiology of avian haematozoa: *Plasmodium*, *Leucocytozoon*, and *Haemoproteus*. In: Loye JE, Zuk M (eds) Bird-Parasite Interactions: Ecology, Evolution, and Behaviour. Oxford University Press, London, pp 19-48

Balenger SL, Johnson LS, Mays HL, Masters BS (2009a) Extra-pair paternity in the socially monogamous mountain bluebird *Sialia currucoides* and its effect on the potential for sexual selection. J Avian Biol 40:173-180

Balenger SL, Johnson LS, Masters BS (2009b) Sexual selection in a socially monogamous bird: male color predicts paternity success in the mountain bluebird. Behav Ecol Sociobiol 63:403-411

Bensch S, Hellgren O, Pérez-Tris J (2009) MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. Mol Ecol Resour 9:1353-1358

Chiver I, Stutchbury BJM, Morton ES (2008) Do male plumage and song characteristics influence female off-territory forays and paternity in the hooded warbler? Behav Ecol Sociobiol 62:1981-1990

Clayton DH (1991) The influence of parasites on host sexual selection. Parasitol Today 7:329-334

Dein FJ (1984) Laboratory Manual of Avian Hematology. Association of Avian Veterinarians, East Northport, New York

Dickinson JL (2001) Extrapair copulations in western bluebirds (*Sialia mexicana*): female receptivity favors older males. Behav Ecol Sociobiol 50:423-429

Dickinson JL (2003) Male share of provisioning is not influenced by actual or apparent loss of paternity in western bluebirds. Behav Ecol 14:360-366

Dickinson JL, Kraaijeveld K, Smit-Kraaijeveld F (2000) Specialized extrapair mating display in western bluebirds. Auk 117:1078-1080

Duckworth RA (2006) Behavioral correlations across breeding contexts provide a mechanism for a cost of aggression. Behav Ecol 17:1011-1019

Fair JM, Myers OB (2002a) Early reproductive success of western bluebirds and ash-throated flycatchers: a contaminated landscape perspective. Environ Pollut 118:321-330

Fair JM, Myers OB (2002b) The ecological and physiological costs of lead shot and immunological challenge to developing Western Bluebirds. Ecotoxicology 11:199-208

Fair JM, Myers OB, Ricklefs RE (2003) Immune and growth response of western bluebirds and ash-throated flycatchers to soil contaminants. Ecol Appl 13:1817-1829

Fair JM, Paul E, Jones J, Clark AB, Davie C, Kaiser G (2010) Guidelines to the Use of Wild Birds in Research. The Ornithological Council, Washington, D.C.

Fair JM, Whitaker SJ (2008) Avian cell-mediated immune response to drought. Wilson J Ornithol 120:813-819

Faircloth BC, Keller GP, Nairn J, Palmer WE, Carroll JP, Gowaty PA (2006) Tetranucleotide microsatellite loci from eastern bluebirds *Sialia sialis*. Mol Ecol Notes 6:646-649

Ferree ED, Dickinson JL, Kleiber D, Stern CA, Haydock J, Stanback MT, Schmidt V, Eisenberg L, Stolzenburg C (2008) Development and cross-species testing of western bluebird (*Sialia mexicana*) microsatellite primers. Mol Ecol Resour 8:1348-1350

Folstad I, Karter AJ (1992) Parasites, bright males, and the immunocompetence handicap hypothesis. Am Nat 139:603-622

Forsman AM, Vogel LA, Sakaluk SK, Thompson CF (2008) Immune-challenged house wren broods differ in the relative strengths of their responses among different axes of the immune system. J Evol Biol 21:873-878

638 Fox A, Hudson PJ (2001) Parasites reduce territorial behaviour in red grouse (*Lagopus lagopus*
639 *scoticus*). Ecol Lett 4:139-143
640 French SS, Moore MC, Demas GE (2009) Ecological immunology: the organism in context.
641 Integr Comp Biol 49:246-253
642 Getty T (2002) Signaling health versus parasites. Am Nat 159:363-371
643 Girard J, Goldberg T, Hamer GL (2011) Field investigation of innate immunity in passerine birds
644 in suburban Chicago, Illinois, USA. J Wildlife Dis 47:603-611
645 Graham AL, Allen JE, Read AF (2005) Evolutionary causes and consequences of
646 immunopathology. Annu Rev Ecol Evol S 36:373-397
647 Graham AL, Shuker DM, Pollitt LC, Auld SKJR, Wilson AJ, Little TJ (2011) Fitness
648 consequences of immune responses: strengthening the empirical framework for
649 ecoimmunology. Funct Ecol 25:5-17
650 Griffith SC, Owens IPF, Thuman KA (2002) Extra-pair paternity in birds: a review of
651 interspecific variation and adaptive function. Mol Ecol 11:2195-2212
652 Guinan JA, Gowaty PA, Eltzroth EK (2008) Western Bluebird (*Sialia mexicana*). In: Poole A
653 (ed) The Birds of North America Online. Cornell Lab of Ornithology, Ithaca, New York
654 Retrieved from the Birds of North America Online:
655 http://bna.birds.cornell.edu/bna/species/510doi:10.2173/bna.510
656 Hamilton WD, Zuk M (1982) Heritable true fitness and bright birds: a role for parasites? Science
657 218:384-387
658 Hanssen SA, Hasselquist D, Folstad I, Erikstad KE (2004) Costs of immunity: immune
659 responsiveness reduces survival in a vertebrate. Proc R Soc Lond B 271:925-930

660 Jacobs AC (2013) Parasites, Immune Response, and Sexual Selection in Western Bluebirds.
661 Dissertation, University of California
662 Jacobs AC, Reader LL, Fair JM (2013) What determines the rates of double brooding in the
663 western bluebird? Condor 115:386-393
664 Jacobs AC, Zuk M (2012) Parasites and Sexual Selection: Do Mechanisms Matter? In: Demas
665 GE, Nelson RJ (eds) Eco-Immunology. Oxford University Press, New York, pp 497-529
666 Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program CERVUS
667 accommodates genotyping error increases success in paternity assignment. Mol Ecol
668 16:1099-1106
669 Kim S-Y, Fargallo JA, Vergara P, Martinez-Padilla J (2013) Multivariate heredity of melanin-
670 based coloration, body mass and immunity. Heredity 111:139-146.
671 Kozma JM, Kroll AJ (2010) Nest survival of western bluebirds using tree cavities in managed
672 ponderosa pine forests of central Washington. Condor 112:87-95
673 Lessells CM, Boag PT (1987) Unrepeatable repeatabilities: a common mistake. Auk 104:116-
674 121.
675 Liebl AL, Martin LB (2009) Simple quantification of blood and plasma antimicrobial capacity
676 using spectrophotometry. Funct Ecol 23:1091-1096
677 Lindström K, Lundström J (2000) Male greenfinches (*Carduelis chloris*) with brighter ornaments
678 have higher virus infection clearance rate. Behav Ecol Sociobiol 48:44-51
679 Martinsen ES, Perkins SL, Schall JJ (2008) A three-genome phylogeny of malaria parasites
680 (*Plasmodium* and closely related genera): Evolution of life-history traits and host
681 switches. Mol Phylogenet Evol 47:261-273

682 Merino S, Moreno J, Sanz JJ, Arriero E (2000) Are avian blood parasites pathogenic in the wild?
683 A medication experiment in blue tits (*Parus caeruleus*). Proc R Soc Lond B 267:2507-
684 2510
685 Millet S, Bennett J, Lee KA, Hau M, Klasing KC (2007) Quantifying and comparing constitutive
686 immunity across avian species. Dev Comp Immunol 31:188-201
687 Møller AP (1990) Effects of a haematophagous mite on the barn swallow (*Hurundo rustica*).
688 Evolution 44:771-784
689 Møller AP (1991) Parasite load reduces song output in a passerine bird. Anim Behav 41:723-730
690 Møller AP, Christe P, Lux E (1999) Parasitism, host immune function, and sexual selection. Q
691 Rev Biol 74:3-20
692 Mougeot F, Evans SA, Redpath SM (2005) Interactions between population processes in a cyclic
693 species: parasites reduce autumnal territorial behaviour of male red grouse. Oecologia
694 144:289-298
695 New Mexico Incident Information System [online] (2011) *Las Conchas*.
696 <http://www.inciweb.org/incident/2385/> (May 11 2012)
697 Nolan PM, Hill GE, Stoehr AM (1998) Sex, size, and plumage redness predict house finch
698 survival in an epidemic. Proc R Soc Lond B 265:961-965
699 O'Brien EL, Dawson RD (2011) Plumage color and food availability affect male reproductive
700 success in a socially monogamous bird. Behav Ecol 22:66-72
701 Palacios MG, Cunnick JE, Winkler DW, Vleck CM (2012) Interrelations among immune
702 defense indices reflect major components of the immune system in a free-living
703 vertebrate. Physiol Biochem Zool 85:1-10

704 Pérez-Tris J, Hasselquist D, Hellgren O, Krizanauskiene A, Waldenström J, Bensch S (2005)
705 What are malaria parasites? Trends Parasitol 21:209-211
706 Råberg L, Stjernman M (2003) Natural selection on immune responsiveness in blue tits *Parus*
707 *caeruleus*. Evolution 57:1670-1678
708 Raouf SA, Parker PG, Ketterson ED, Nolan V Jr, Ziegenfus C (1997) Testosterone affects
709 reproductive success by influencing extra-pair fertilizations in male dark-eyed juncos
710 (Aves: *Junco hyemalis*). Proc R Soc Lond B 264:1599-1603
711 Ricklefs RE, Fallon SM (2002) Diversification and host switching in avian malaria parasites.
712 Proc R Soc Lond B 269:885-892
713 Salvante KG (2006) Techniques for studying integrated immune function in birds. Auk 123:575-
714 586
715 SAS Institute (2014) JMP Version 11. SAS Institute Inc, Cary, North Carolina
716 Schmid-Hempel P (2003) Variation in immune defense as a question of evolutionary ecology.
717 Proc R Soc Lond B 270:357-366
718 Schmid-Hempel P (2008) Parasite immune evasion: a momentous molecular war. Trends Ecol
719 Evol 23:318-326
720 Sheldon BC, Verhulst S (1996) Ecological immunity: costly parasite defense and trade-offs in
721 evolutionary ecology. Trends Ecol Evol 11:317-321
722 Shizuka D, Dickinson JL (2005) Using molt limits to age western bluebirds. J Field Ornithol
723 76:193-196
724 Siefferman L, Hill GE (2003) Structural and melanin coloration indicate parental effort and
725 reproductive success in male eastern bluebirds. Behav Ecol 14:855-861

726 Siefferman LM, Hill GE (2005) Blue structural coloration of male eastern bluebirds *Sialia sialis*
727 predicts incubation provisioning to females. J Avian Biol 36:488-493
728 Stjernman M, Råberg L, Nilsson J (2008) Maximum host survival at intermediate parasite
729 infection intensities. PLoS ONE 3:e2463
730 Viney ME, Riley EM, Buchanan KL (2005) Optimal immune responses: immunocompetence
731 revisited. Trends Ecol Evol 20:665-669
732 Waldenström J, Bensch S, Hasselquist D, Östman Ö (2004) A new nested polymerase chain
733 reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections
734 from avian blood. J Parasitol 90:191-194
735 Wilcoxen TE, Boughton RK, Schoech SJ (2010) Selection on innate immunity and body
736 condition in Florida scrub-jays throughout an epidemic. Biol Lett 6:552-554
737 Zahavi A (1975) Mate selection: a selection for a handicap. J Theor Biol 53:205-214
738 Zahavi A (1977) The cost of honesty (further remarks on the handicap principle). J Theor Biol
739 67:603-605
740 Zehtindjiev P, Ilieva M, Westerdahl H, Hansson B, Valkiūnas G, Bensch S (2008) Dynamics of
741 parasitemia of malaria parasites in a naturally and experimentally infected migratory
742 songbird, the great reed warbler *Acrocephalus arundinaceus*. Exp Parasitol 119:99-110
743 Zuk M, Stoehr AM (2002) Immune defense and host life history. Am Nat 160:S9-S22
744 Zuk M, Thornhill R, Ligon JD, Johnson K (1990) Parasites and mate choice in red jungle fowl.
745 Am Zool 30:235-244
746

747 Table 1 Results of the logistic regression performed to determine whether individual traits
748 affected the probability that a male would sire extra-pair offspring. We considered mass,
749 breeding date (as indicated by the hatch date of the nestlings), infection status (0=uninfected,
750 1=infected), immune response (bactericidal capacity of the blood/BKA), and age as predictor
751 variables

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

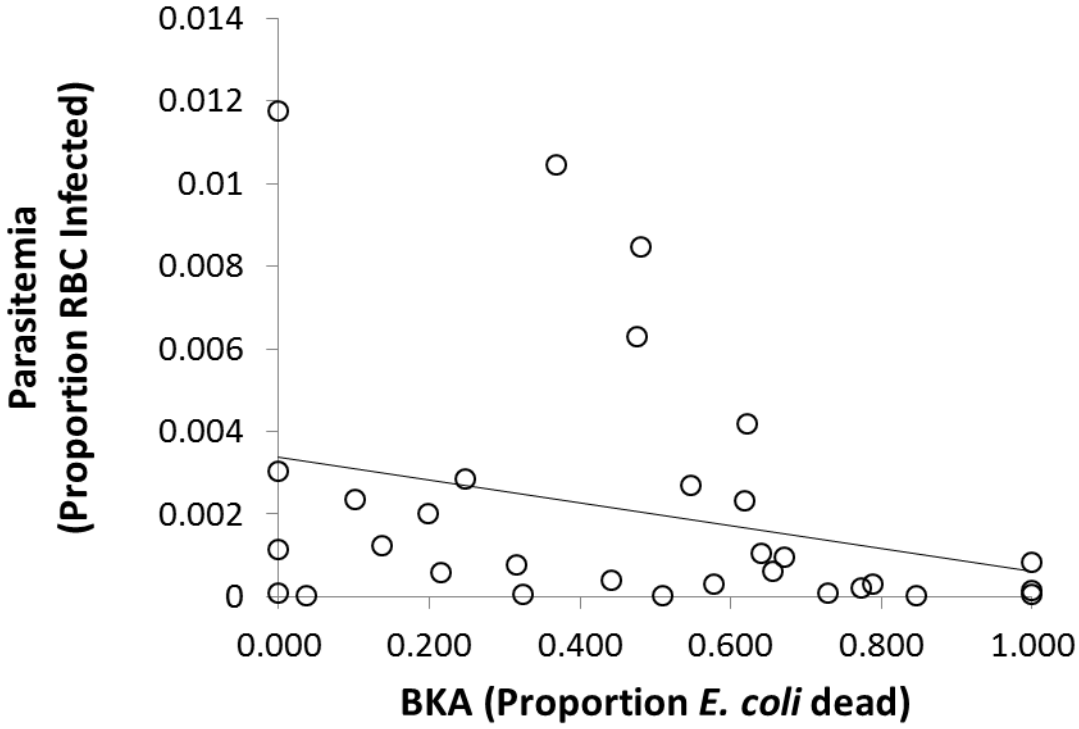
752

Figure Legends

Figure 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

Figure 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

762 FIGURE 1



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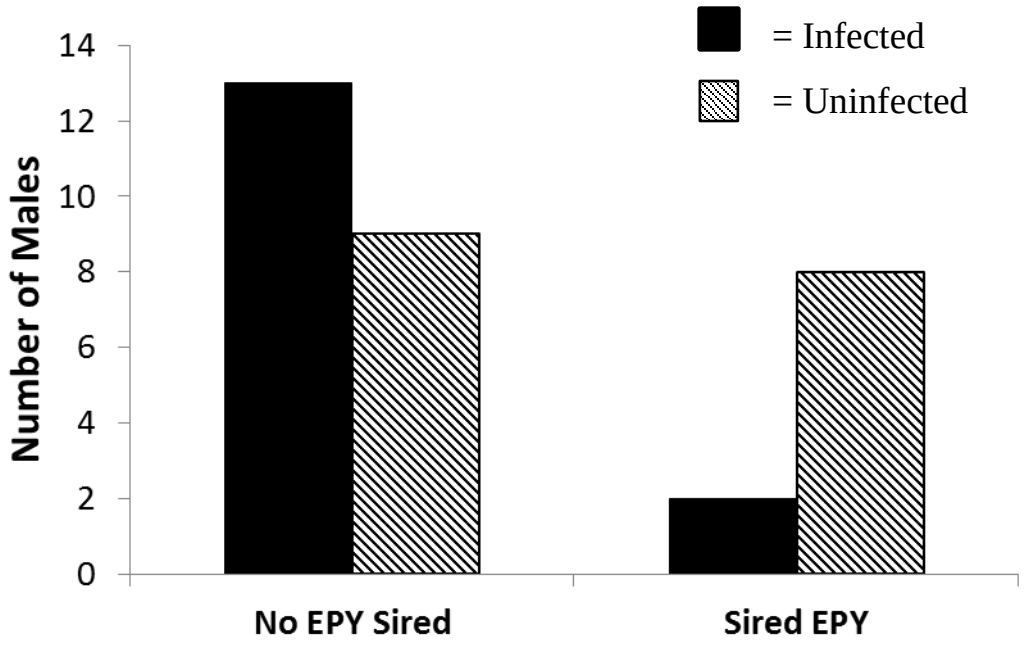
767 FIGURE 2



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