Female Mating Preference and Reproductive Success in Eastern Bluebirds: Interacting Effects of Plumage Coloration and Genetic Compatibility

by

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Abstract

In my dissertation, I used a combination of aviary-based experiments, field experiments and correlational research to fully explore the importance of mate choice and environmental heterogeneity on reproductive success in eastern bluebirds. In the first half of my dissertation (chapters 1, 2, and 3), I used aviary and field manipulations to tests the hypotheses that female mating preferences are based on male plumage ornamentation and genetic characteristics. In the second half of dissertation (chapters 4 and 5), I examined whether male characteristics and environmental factors influence reproductive success.

In the first chapter, I examined female mating preferences in eastern bluebirds by utilized aviary-based mate-choice experiments. I found no evidence that female eastern bluebird chose mates based on their structural coloration.

In the second chapter, I used a field-based design to again test female mate preferences for male ornamentation. To control for the influence of territory quality on female choice, I widowed dyads of males with adjacent territories. I found the more-ornamented and larger males did not attract females more quickly compared to the less-ornamented and smaller males, thus I found no evidence that females preferentially settled with the more-ornamented males.
In third chapter, I used the same experimental design and tested the importance of male heterozygosity and the genetic compatibility of the pair on female preferences in the wild. I found that females preferred to pair with the most genetically compatible mates. In the fourth and fifth chapters, I focused on reproductive success of wild breeding eastern bluebirds.

In the fourth chapter, I found that both male coloration and genetic compatibility of pairs predicted offspring quality. The relative importance of genetic compatibility versus mate ornamentation on nestling quality, however, varied with season.

In the fifth chapter, I explored environmental factors that influence hatching failure. I found that the relative humidity measured from the nearest weather station was positively related to the likelihood that eggs would fail to hatch. I found no consistent relationship, however, between seasonality and hatching failure, nor did I find that pre-incubation nest temperatures predicted hatching failure.
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Chapter 1. An Experimental Test of Female Choice Relative to Male Structural Coloration in Eastern Bluebirds
Abstract

Several experimental studies have shown that female birds use ornamental melanin and carotenoid plumage coloration as criteria in mate choice. Whether females choose mates based on natural variation in structural coloration, however, has not been well established. Male eastern bluebirds (*Sialia sialis*) display brilliant ultraviolet (UV)-blue plumage coloration on their head, back, wings, and tail that is positively correlated with condition, reproductive effort, and reproductive success. We experimentally tested the hypothesis that female eastern bluebirds prefer as mates males that display brighter structural coloration by presenting breeding-condition females with males of variable coloration. We conducted two types of mate choice experiments. First, females chose between males whose coloration was manipulated within the natural range of variation in the population; feathers were either brightened with violet marker or dulled with black marker. Second, females chose between males with naturally dull or bright plumage coloration. In both manipulated and unmanipulated coloration trials, female choice did not differ significantly from random with respect to structural coloration. We found no support for the hypothesis that the UV-blue coloration of male eastern bluebirds functions as a criterion in female mate choice.
Introduction

Darwin (1871) first proposed that the bright ornamental coloration displayed by males of many animal species evolved in response to female mate preferences for brightly colored males. The hypothesis that females prefer as mates males with the most elaborate expression of color displays has now been experimentally tested in several species of passerine birds relative to expression of both the color quality of red and yellow carotenoid plumage coloration and the size of black eumelanin feather patches (Hill 2006). For both types of color display, convincing evidence has been published for female choice for extreme expression of color display within the natural bounds of color expression (summarized in Hill 2006).

Mate choice relative to expression of structural plumage coloration has received much less study than mate choice for pigment-based coloration. Variation in non-iridescent structurally-based coloration results from coherent scattering of light from the spongy layer of feather barbs (Shawkey et al. 2003; Prum 2006) and this type of microstructure is often responsible for green, blue, and violet coloration. Most structural plumage coloration also has a substantial reflection in the ultraviolet (UV) region of the spectrum. By definition, UV light is invisible to humans, but it is part of the perceptual color space of diurnal birds (Cuthill 2006). UV coloration, therefore, must be taken into account in studies of the function of structural coloration (Bennett et al. 1997).
Most mate choice studies that involve structural feather coloration have been conducted by removing UV reflectance. These studies eliminated UV reflectance by either placing males behind UV blocking windows (Maier 1993; Bennett et al. 1996; Hunt et al. 1997; Hunt et al. 1999) or by applying UV blocking chemicals to plumage (Andersson and Amundsen 1997; Siitari and Huhta 2002). In all studies, females discriminated against males that appeared to lack UV reflectance. These studies show that females see the UV component of feather coloration and avoid odd looking males with no UV component to their plumage, but they do not test for preferences relative to natural variation in structural plumage coloration (Hill 2006).

In contrast to extensive literature on female choice relative to pigment-based coloration, only four experimental studies of female mate choice relative to natural variation in structural plumage coloration have been conducted. Bennett et al. (1997) tested female preference relative to natural variation in the iridescent structural coloration of males of European starling (*Sturnus vulgaris*), and found that females showed an association preference for males that exhibited coloration with greater reflectance in violet and red regions. Likewise, female blue tits (*Parus caeruleus*) prefer males with brighter non-iridescent UV-blue structural plumage (Hunt et al. 1998). Only two studies have manipulated structural coloration within natural variation to date. Ballentine and Hill (2003) manipulated the plumage of male blue grosbeaks (*Passerina caerulea*) to move them to the extremes of color expression in the wild population and found that the patterns
of female choice relative to male color display did not differ significantly from random. By
manipulating UV reflectance of male bluetits (*Parus caeruleus*), Johnsen et al. (2005) found that
females adjust parental effort in response to male appearance.

Manipulation of structural coloration within a natural range of expression represented an
improvement over previous studies that used UV-blocking techniques in two important ways.
First, such an experiment assesses female response to male coloration within a range of color
displays that would occur among males in nature and thus places the mate choice experiment with
a relevant context. Second, by manipulating male coloration, the researcher is able to randomize
plumage coloration with respect to all potential confounding variables (e.g. song, behavior, health,
vigor) and in this way conduct a more convincing test of the focal trait.

We tested whether female eastern bluebirds (*Sialia sialis*) use expression of UV-blue
structural coloration of males as a criterion of mate choice. Eastern bluebirds are socially
monogamous and sexually dimorphic passerines that breed throughout eastern North America
(Gowaty and Plissner 1998). Males display conspicuous UV-blue structural coloration on their
head, back, rump, tail and wings. The coloration has a spectral reflectance peak that corresponds
to approximately 400nm and reflects UV and blue wavelengths of light equally (Siefferman and
Hill 2003). Field studies show that males with brighter structural coloration gain a reproductive
advantage. A field correlation study demonstrates that more colourful males paired earlier in the
season and experienced greater reproductive success than less colourful males (Siefferman and Hill 2003). Field experiments indicate that more colourful males are better able to gain access to limited nesting resources (Siefferman and Hill 2005b). These patterns could result from brighter males either being more attractive to females during mate choice or gaining an advantage in competition with other males for resources.

The goal of this study was to test whether UV-blue coloration of males serves as an important criterion in female mate choice. We conducted our experiments in an aviary so that we could control male-male interactions and manipulate the coloration of males. We tested female choice for male coloration in two experiments. First, we manipulated the plumage coloration of stimulus males within the natural range of variation in the population to investigate whether females preferred brighter males. Second, we presented females with males with naturally bright or drab structural coloration. Because prior field work showed that males that display brighter structural coloration pair earlier, we predicted that in both manipulated and unmanipulated plumage coloration trials females would prefer brighter males.

Methods

During the spring of 2002 and 2004, we used mist nets to capture adult eastern bluebirds from three different locations in Macon and Lee Counties, Alabama, USA. All birds were in adult
breeding plumage but were of unknown age. We housed birds in uni-sex flocks of 5-8 birds per cage (2.5mx1.5mx5m) at an aviary on the campus of Auburn University. Prior to and during the experiment, captive birds were provided with water and a diet of meal worms (Tenebrio molitor), crickets (Acheta domesticus), and wax worms (Galleria mellonella) ad libitum.

Plumage measurements

We collected eight feathers from the rump of each male and taped the feathers on black paper (Canson® Cat: #425 Stygian black) overlapping to recreate a colored patch. We measured the plumage reflectance using an Ocean Optics S2000 spectrometer and deuterium tungsten halogen light source (range 250-880nm: Dunedin, Florida, USA). All measurements were taken perpendicular to the feather surface using a metal fiber optic probe mount with a rubber cap to exclude ambient light. We set the distance between the probe and feather at 5mm to create a 2mm measurement area. We generated reflectance data relative to a white standard (Labsphere, Inc.®) using OOIBase software (Ocean Optics®). We took five measurements from each feather sample and averaged them to represent the UV-blue coloration of each male.

We summarized reflectance data by calculating three standard descriptors of reflectance spectra: brightness, chroma, and hue. Mean brightness was calculated as the mean summed reflectance (R300-700nm). UV chroma was calculated as the proportion of the total reflectance
Blue chroma was calculated as the proportion of the total reflectance (R_{300-700nm}) that is in the blue part of the spectrum (R_{400-500-nm}). Hue was calculated as the wavelength at peak reflectance. These calculations taken from the same spectral curves are correlated in eastern bluebirds (Siefferman et al. 2005) such that the most ornamented males display brighter coloration and greater UV chroma. Because past experiments have demonstrated that brightness of structural coloration but not chroma or hue is condition dependent (Siefferman and Hill 2005a), we chose to manipulate the brightness of male plumage coloration. Because we only took spectral measurements of each manipulated bird in 2004, some analyses only include the 2004 data set.

**General mate choice methods**

To be certain that the females did not choose between males with whom they might have had prior experience, we conducted each trial with males and females that were captured at different study areas. We conducted mate choice trials in two large outdoor mate-choice arenas (6mx8mx2m; Fig. 1) under natural light conditions. At the beginning of each mate choice trial, we placed two males in adjacent cages that were separated by plywood. We placed a female in a third compartment that spanned the entire length of the aviary and allowed her visual and acoustic but no physical contact with the males. To facilitate natural breeding conditions and mate choice,
we placed nest-boxes between the female cage and both male compartments. The nest-boxes were designed with two entrances so that males and females could enter the box freely, but birds were separated in the box by a mesh barrier (Saetre et al. 1994). All cages had many perches, and we positioned two “display perches” for the female directly in front of each male cage, approximately 0.2m from the male cage. We used these “display perches” to calculate association time (Fig 1). Females were allowed to choose between two simultaneously displaying males. Females also had access to a neutral area from which she had no visual contact with either male. Immediately after we placed birds into the mate choice arena, we videotaped (Sony Hi-8) the behavior of all three birds for 3 continuous hrs. In nearly all trials, within 15min of the commencement of the trials, males began to sing and display from the perches. After each trial, birds were released to their original capture location and several birds bred successfully later in the season.

In 2002 and 2004, we manipulated plumage coloration of males for mate choice trials by enhancing or reducing plumage coloration. Males were assigned to treatment groups by pairing males of similar body size and natural plumage coloration and then randomly assigning males to treatment groups. We used non-toxic black permanent marker (Sharpie® permanent marker: black) to decrease brightness of UV-blue colour and violet permanent marker (PRISMACOLOR® PM-60: violet mist) to enhance brightness of UV-blue colour. Note that because coloration of
male bluebirds results from microstructure and not pigments, feathers colored with a black marker still looked blue to a human observer and the reflectance from such feathers still had a spectral shape characteristic of blue (Fig. 2). The black ink from the pen uniformly absorbed a percentage of light reaching the microstructures, uniformly reducing the brightness of coloration.

We colored all blue feathers on the head, neck back, rump, tail and wings of each male carefully. Because the marker temporarily wetted the feathers and changed the brightness of feathers perceptibly, we were certain that we applied even coloration across the feathered surface of the bird. The markers dried quickly and did not cause the birds to look abnormal to the human observer. Markers were applied 10 min before the commencement of each trial.

Ideally, in a mate choice experiment, each experiment would be conducted with a different set of stimulus males to avoid any possibility of pseudoreplication creating or obscuring patterns. In our experiment in which we tested up to 46 females in each experiment, this would have required maintaining 96 males in captivity. It was not feasible, and we felt not ethical, to capture such a large number of males. As a compromise, we used 16 different males to generate 24 dyads of males in 2002 and 21 different males to generate 26 dyads of males in 2004. Males were randomly assigned to a color manipulation and males in the two groups did not differ significantly in size or pre-manipulation plumage coloration (Table 1). Three criteria were used when choosing dyads of males for mate choice trials. First, males must have been captured from
different field site than females. Second, males were assigned to dyads such that each dyad was
represented a unique pair of males. Finally, within this subset of available males, we chose males
randomly from large flight cages. We used a unique female for each mate choice trial within an
experiment. In 2004, we used 16 of the same females for the natural coloration experiment and
the manipulated coloration experiment.

We also conducted mate choice trials in which females were given the opportunity to choose
between two males of natural plumage coloration. First we measured plumage coloration, then
we formed each trial by creating unique dyads by pairing males of similar body size that differed
by at least 20% in brightness of their plumage. Early in the breeding season of 2004, we used 20
males to create 20 unique dyads of males and assigned a different female to each of the unique
dyas.

Data analyses

We transcribed the video footage recorded for 2 continuous hrs between 0.5 to 2.5 hrs after the
commencement of trials. We began transcribing footage at 0.5 hrs after the commencement of
the trials to allow the birds to acclimate to their surroundings. Females had the opportunity to
perch directly in front of males on the “display perches” or on many other perches. We defined
total association time as the total time that the female perched on either of the “display perches”
during the 2 hr trial. As our index of preference, we calculated the proportion of time that a female associated with each male. We considered a trial to be a successful choice if the female spent $\geq 60\%$ of her association time with one male. We conducted all analyses using JMP 4.0.1 (SAS Institute Inc.). We tested for normality using Shapiro-Wilk tests, using parametric tests when data were normally distributed and non-parametric tests when assumptions of normality were violated.

Results

Color manipulation

The permanent marker treatments effectively changed the plumage coloration of male eastern bluebirds (Table 1, Fig. 2). By comparing the treatment with natural coloration of males taken over the course of four breeding seasons, we found that the resulting plumage coloration was within the natural range of variation in the breeding population (Table 1). The plumage brightness of one male, however, was manipulated below the natural range of variation. Although, the following analyses were conducted while both including and excluding the trials in which this male was used; the test results were similar. In general, the manipulation affected the overall brightness but changed the shape of the spectral curve very little and there was no significant effect on hue. Although the proportional UV and blue reflectance (chroma) were
influenced by the manipulation (Table 1), this is mainly because the manipulations altered the reflectance between 300 and 600 nm without altering the reflectance in the longest wavelengths (R_{600-700nm}; see Fig. 2).

*Mate choice experiment: general responsiveness*

Both male and female bluebirds interacted during trials in a manner that suggested they were receptive to forming pairs and mating. During trials, males were active, usually sang typical songs and often made high pitched ‘courtship’ vocalizations, fanned their tails, and displayed their rumps to females. In turn, females interacted with males via vocalizations and inspecting nest boxes. Some females even began building nests.

*Mate choice experiment: manipulated plumage*

We conducted 24 trials in 2002 and 26 trials in 2004; however one female in 2002 and three females in 2004 did not make a clear choice of mate. We found no differences in the preferences for duller and brighter males according to year ($\chi^2 = 2.13, df = 1, P = 0.14$), therefore we combined the data from 2002 and 2004. We found no evidence that females preferred brighter males; 23 females preferred males that exhibited increased plumage coloration, and 23 females preferred males that exhibited decreased plumage coloration ($\chi^2 = 0.00, df = 1, P = 1.00$; Fig. 3a). A subset
of females spent time in nest boxes. Again, we found no evidence that females preferred brighter males; 11 females that visited nest boxes preferred brighter males and nine females that visited nest boxes preferred males that exhibited duller plumage coloration ($\chi^2 = 0.20, df = 1, P = 0.66$).

Three females initiated nest building in nest boxes. Again, we found no evidence that females preferred brighter males; one female that a built nest preferred brighter males and two females that built nests preferred males that exhibited duller plumage coloration.

We used linear regression, to determine whether the strength of the females’ preference was influenced by the difference in colour of two males in the dyads. We found no significant relationships between female association time and relative difference in plumage brightness ($R^2 < 0.01, F_{1,23} = 0.03, P = 0.86$; Fig. 4), UV chroma ($R^2 < 0.01, F_{1,23} < 0.01, P = 0.99$), blue chroma ($R^2 < 0.01, F_{1,23} = 0.01, P = 0.91$), or hue ($R^2 < 0.01, F_{1,23} < 0.01, P = 0.94$).

**Mate choice trials using natural coloration**

We conducted 20 mate choice trials in which females chose between two males with unmanipulated plumage coloration. In one of these trials a female did not spend $\geq 60\%$ of their time with one male and thus, by our criteria, made no obvious mate choice decision. Of the 19 trials in which females made definite mate-choice decisions, we found no evidence that females preferred brighter males; 11 females preferred males that exhibited brighter coloration and eight
females preferred males that exhibited duller coloration ($\chi^2 = 0.49$, $df = 1$, $P = 0.47$; Fig. 3b).

Moreover, we used paired t-tests to compare the colour characteristics of the winners and losers of each trial. Again, we found no significant difference in the plumage brightness ($t = 0.20$, $n = 19$, $P = 0.84$), UV-chroma ($t = -0.18$, $n = 19$, $P = 0.86$), blue-chroma ($t = -1.14$, $n = 19$, $P = 0.27$), or hue ($t = 0.07$, $n = 19$, $P = 0.94$) of males that were preferred and not preferred. Further, because dyads varied in degree of difference in coloration between the brighter and duller males, we used linear regression to investigate whether there were relationships between the strength of female preference and the difference in the plumage coloration of males. We found no significant relationships between female association time and relative difference in plumage brightness ($R^2 = 0.04$, $F_{1,18} = 0.7$, $P = 0.41$), UV chroma ($R^2 < 0.01$, $F_{1,18} = 0.09$, $P = 0.77$), blue chroma ($R^2 = 0.12$, $F_{1,18} = 2.53$, $P = 0.13$), or hue ($R^2 = 0.02$, $F_{1,18} = 0.33$, $P = 0.57$).

We calculated the power of our manipulated mate choice experiment ($Power = 0.98$, $n = 46$) by using previous studies that found that $\geq 80\%$ of females preferred more ornamented males.

Lastly, by combining data from all of the mate choice experiments ($n = 65$), we tested whether females displayed a preference for one side of the mate choice chamber. We found no differences in the preferences for males on the right or left side of the chamber ($\chi^2 = 1.86$, $df = 1$, $P = 0.17$).

*Consistency of choice*
Although each trial represented a unique trio of birds (1 female and 2 males), in some cases, males were used more than one time, and 16 of the females that were used in the artificially manipulated coloration trials were also used in the natural coloration trials. We investigated whether individual males were consistently chosen by females. In the mate choice experiment in which females were given the opportunity to choose between 2 males of manipulated plumage coloration, 13 of the 17 (76%) males were not consistently chosen or not chosen at all by females. In natural coloration trials, 10 of 14 (71%) males that were used in multiple trials were not consistently chosen or not chosen at all by females. Moreover, when males were used in more than one trial, we calculate a winning rate for each male (# winning trials / total # trials). We used linear regression to test whether the proportional winning rate was related to spectral measures of manipulated plumage coloration. The winning rate was not significantly related to the brightness, UV chroma, blue chroma, or hue of the structural coloration (all \( P > 0.1 \)). Sixteen females were used in both manipulated and natural plumage coloration trials. Females did not consistently choose either the brighter or duller male (\( \chi^2 = 0.25, df = 1, P = 0.62 \)).

Discussion

We found no support for the hypothesis that female eastern bluebirds prefer to mate with males with more elaborate structural ornamental plumage coloration, regardless of whether we
conducted trials with males whose plumage was manipulated or not manipulated. Females showed no preferences for males displaying brighter blue feathers or blue feathers with greater UV chroma, greater blue chroma, or more UV-shifted hues. These results were surprising because prior field correlations indicated that structural coloration of males is a sexually selected trait that reliably conveys information about male condition (Siefferman and Hill 2005a), age (Siefferman et al. 2005) and breeding success (Siefferman and Hill 2003). Negative results for mate choice are only convincing if experiments have sufficient power and if test animals are receptive to stimulus animals. Our sample size was relatively large for a mate choice experiment and we observed the same indifference to color display in two separate mate choice experiments. Many experiments assessing choice of mates based on expression of carotenoid or eumelanin coloration and using an aviary design similar to what we used have found positive results with many fewer trials (Hill 1990; Sundberg 1995; Kimball 1996).

Our findings are also unlikely to simply be an effect of captivity. All of the females used in this experiment were in reproductive condition, and we are confident that females were actively choosing males. Behavioral observations during trials indicate that both males and females displayed courtship behaviors and that females often inspected nest boxes. Indeed, if association preference is considered a weak measure of mate preference, we can use box visitation, which is a much less ambiguous indicator of preference. For the subset of females that visited boxes, mate
choice was random with respect to plumage coloration. We assert that our mate choice trials represent convincing evidence that female bluebirds were not using variation in brightness or chroma of structural coloration to choose males. Our experiment, however, did not quantify or manipulate the melanin-based breast coloration of the eastern bluebird. It is possible that mate choice decisions of females are influenced by the patch size or coloration of the orange breast plumage. Indeed in past research, using a composite color score including variation in both melanin and structural coloration, we found that males with larger, darker melanin patches and brighter blue-UV coloration fed chicks more often and were mated to females that begin laying eggs earlier in the season (Siefferman and Hill 2003). Moreover, we did not measure or manipulate other variables, such as song and age that may also have influenced association preferences.

In addition to female choice, male-male competition, and a combination of male-male competition and female choice can drive the evolution of ornamental traits in many species (Andersson 1994; Berglund et al. 1996). Environmental constraints often influence the evolution of male-male competition versus female preferences in animal mating systems (Reynolds 1996). For instance, when breeding sites are readily defensible, male-male competition is probably more important than female choice in influencing male reproductive success. Bluebirds are obligate secondary cavity nesters and male and female bluebirds compete vigorously against same-sex
conspecifics for nest sites (Gowaty and Wagner 1988). It may be that structural coloration in eastern bluebirds functions a reliable indicator of male competitive ability and is used in mediating contests over nests sites. Indeed, an experiment in which the settlement pattern of bluebirds was assessed relative to male plumage coloration indicated that male plumage coloration was a good predictor of male resource-holding potential (Siefferman and Hill 2005b).

In the field we have observed relationships between the plumage coloration of male eastern bluebirds and first egg date of females, paternal feeding rates, and offspring condition. These patterns could be used as indirect evidence that females use male coloration in choosing mates. Such patterns, however, are also consistent with structural coloration serving as a signal primarily in male-male contests. If structural coloration functions in male-male competition for access to high quality territories, brighter males may pair earlier, feed offspring more often, and experience greater reproductive success because insect abundance is higher in their territories. Perhaps females choose mates by assessing territory quality and not plumage coloration. Our studies of eastern bluebirds illustrate how very difficult it can be to tease apart the potential roles of male-male competition and female choice of male in the field and hence the value of controlled aviary mate-choice experiments.
Acknowledgements

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Reference


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Table 1.1
Plumage coloration of male eastern bluebirds used in the 2004 mate choice manipulation. Plumage data were not available for 2002 males. Data include coloration both before and after plumage manipulation as well as body size. Measures are compared using independent t-tests. Pretreatment males were similar in plumage coloration and body size. Natural ranges of original color variables are based on a larger subset of 224 breeding males captured during 2003-2006.

<table>
<thead>
<tr>
<th></th>
<th>Decreased color</th>
<th>Enhanced color</th>
<th>Range of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=8)</td>
<td>(n=10)</td>
<td>t</td>
</tr>
<tr>
<td>Original Brightness</td>
<td>28.35±1.83</td>
<td>31.44±1.63</td>
<td>1.26</td>
</tr>
<tr>
<td>Treatment Brightness</td>
<td>21.24±2.02</td>
<td>32.74±1.80</td>
<td>4.25</td>
</tr>
<tr>
<td>Original UV Chroma</td>
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<tr>
<td>Treatment UV Chroma</td>
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<td>33.53±0.64</td>
<td>5.00</td>
</tr>
<tr>
<td>Original Blue Chroma</td>
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<td>40.56±0.30</td>
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</tr>
<tr>
<td>Treatment Blue Chroma</td>
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<td>41.71±0.29</td>
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</tr>
<tr>
<td>Original Hue (nm)</td>
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<td>415.06±2.85</td>
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</tr>
<tr>
<td>Treatment Hue (nm)</td>
<td>424.00±3.03</td>
<td>417.30±2.71</td>
<td>-1.65</td>
</tr>
<tr>
<td>Wing Chord (mm)</td>
<td>98.22±0.61*</td>
<td>98.78±0.61*</td>
<td>0.64</td>
</tr>
<tr>
<td>Mass (g)</td>
<td>26.88±0.56*</td>
<td>27.93±0.56*</td>
<td>1.34</td>
</tr>
</tbody>
</table>

*n=9
Diagram of the outdoor mate-choice aviary used for the eastern bluebird female choice experiment.

The sexes are separated by central wall (dotted line) made of plywood on the bottom half and wire mesh on the top half; separations between male cages (thin solid lines) are plywood. Outer walls (thick solid lines) are plywood. The roof is made of wire mesh to allow natural light. The female area is separated by a long half-wall (ceiling to 1m from floor, long broken line) made of plywood to allow the females a “neutral area” that contained perches, food, and water and visually separated her from the males. For a female to display mate choice she had to fly underneath the wall where she could view and interact with males in two cages. Nest boxes with double entrances (arrows
point to entrances) were mounted directly below each display perch. “Display perches” (solid line) are near the nest boxes, and when a female sat on these perches we counted her as having visited the male in that cage.
Figure 1.2

Reflectance spectra of plumage coloration showing the effect of applying the enhancing marker and the reducing marker on the rump feathers of male eastern bluebirds. The thick black line is the spectrum after decreased treatment; the thick gray line represents the spectrum after the enhanced treatment, with SD error bars at every 50nm interval. The thin black line represents the natural plumage color before marker treatment; error bars are excluded for clarity. Note: we applied the same color treatment to every blue body region (all parts excluding breast and belly); the markers had a similar influence on all body regions.
(a) Number of Females

Enhanced       Decreased

Plumage color

(b) Number of Females

Natural Bright       Natural Dull

Plumage color
Figure 1.3

Number of female eastern bluebirds that chose brighter or duller males in mate choice trials for a) males displaying manipulated coloration and b) males displaying unmanipulated coloration.
Figure 1.4

Relationship between female association time and relative difference in plumage brightness of males in each dyad. A strength of preference of 1 represents a female that spent all her time with one male.
Chapter 2. A Field Test of Female Mate Preference for Male Plumage Coloration in Eastern Bluebirds
Abstract

A growing body of evidence shows that female birds use male plumage coloration as an important criterion in mate choice. In the field, however, males with brighter coloration may both compete better for high quality territories and be the object of female choice. Positive associations between territory quality, male-male competitive ability, and female preferences can make it difficult to determine whether females actively choose the most ornamented males. Male eastern bluebirds (Sialia sialis) display brilliant ultraviolet (UV)-blue plumage coloration on their heads, backs, wings, and tails, and chestnut coloration on their breasts which is positively correlated with condition, reproductive effort, and reproductive success. We tested the hypothesis that female bluebirds prefer males that display brighter and more chromatic coloration by widowing males in the field and allowing replacement females to choose partners. We controlled for the influence of territory quality on female choice by widowing dyads of males with adjacent territories. We found no evidence that UV-blue or chestnut plumage coloration, body size, or body condition predicted the male with which females would pair. We found no support for the hypothesis that the coloration of male eastern bluebirds functions as a criterion in female mate choice.
Introduction

It is generally thought that the bright plumage coloration displayed by males of many bird species evolved in response to selection from female choice for elaborate displays (Hill 2006). Indicator models of sexual selection propose that the expression of sexually selected traits, such as plumage coloration, reliably signal individual condition (Andersson 1994; Hill 2002). These traits can act as honest signals of an individual’s phenotypic or genetic quality if only exceptionally fit individuals in a population achieve maximum expression of such traits (Zahavi 1975; Hamilton and Zuk 1982; Kodric-Brown and Brown 1984). Indicator models predict that individuals that display more-exaggerated traits compete better for mates and thus experience higher reproductive success. There is now substantial experimental and correlational evidence that, in some species of birds, females discriminate between potential mates by assessing colourful plumages (reviewed in Hill 2006).

Studies of female mate choice in birds range from weak correlative field studies and uncontrolled aviary observations to carefully controlled experimental tests in both the field and the laboratory. Laboratory experiments can be useful because they allow researchers to manipulate coloration and disassociate colour traits from other correlated traits. Eliminating the confounding influence of male-male competition, for example, can be very helpful (Wagner 1998). However, eliminating male-male competitive interactions usually necessitates that males are housed
individually and thus reduces physical access to females. When females do not have physical access to potential mates and cannot copulate with males, researchers generally use association time as a proxy for mate choice. Association time, however, may not always be an accurate and consistent measure of mate preference (Hill 2006). Females also may not make mate choice decisions in the relatively unnatural environment of the laboratory. Finally, in laboratory-based mate choice trials, it becomes difficult for researchers to investigate the fitness consequences of mating decisions (Hill 2006). For example, for most species in the laboratory, it is not possible to examine how the chosen male contributes to the female’s breeding success. Contributions to reproductive success include direct benefits (territory quality and parental care) and indirect benefits (fertilization rate and genetic quality) provided by males. Controlled tests of female preferences conducted in the field provide an alternative to laboratory-based trials and can allow researchers to quantify both female choice and the ramifications of those choices.

The eastern bluebird (*Sialia sialis*) is among the better studied bird species with respect to the function of ornamental plumage coloration, and yet the importance of male coloration as a criterion in female mate choice remains uncertain. Male bluebirds display a structural UV-blue coloration on the plumage of their backs, heads, wings and tails and chestnut melanin coloration on their breasts compared with the overall duller coloration in females. Although no studies have examined visual perception in eastern bluebirds, UV vision has been demonstrated in closely
related species (Hart et al. 2000) and UV-blue coloration in passerine bird has been suggested to play an important role in signal communication (Hill 2006). A correlative field study of eastern bluebirds shows that male eastern bluebirds with brighter UV-blue plumage and darker melanin breast colour pair earlier in the season, feed chicks more often, and gain higher reproductive success (Siefferman & Hill 2003). Experimental manipulations of paternal investment demonstrate that UV-blue structural coloration is a condition-dependent trait in bluebirds (Siefferman & Hill 2005a). Although the field correlative study linking coloration earlier pairing and reproductive success suggests that females may use coloration to choose mates, experimental manipulations of structural coloration in an aviary show no consistent relationship between male UV-blue coloration and female association time (Liu et al. 2007).

Bluebirds are obligate cavity-nesting passerines and males that express more-ornamented UV-blue coloration are more successful competitors for limited nest sites (Siefferman & Hill 2005b). Thus, in the eastern bluebirds as in many species that defend key resources necessary for reproduction, female choice for male coloration is confounded by female choice for the superior resources defended by brighter males. Simple correlative studies cannot distinguish the relative influences of female choice of mate and male-male competition in driving the elaboration of plumage coloration.
To more directly test female preferences for ornamental plumage coloration in a wild population of breeding eastern bluebirds, we conducted a mate removal experiment. We removed the original mates from males to quantify the latency time for males to establish new pair bonds. We used this protocol to avoid any bias associated with prior pair bonds, because past relationships can confound mate choice in two ways. Pairs that have previous breeding experience at the same territory might experience advantages in acquiring nesting sites (Qvarnstrom & Forsgren 1998) and in reproductive success (Ligon 1999). We removed the mates of two males that held neighboring territories with the assumption that males with adjacent territories held territories of similar quality. This experiment was designed to measure female preference in the wild and to quantify the breeding success of the subsequent mate selection on a platform of equal quality territories.

First, by removing original mates early in the breeding season, we caused pairs to reconstruct the early breeding season processes of mate choice and pairing. Territories in which females had been removed represented available males. Second, we investigated the characteristics of the males and determined which traits predicted male attractiveness to females. We tested the hypothesis that female eastern bluebirds prefer to pair with males that display the more-ornamented plumage coloration. Additionally, by monitoring the behaviour of the newly formed pairs throughout the breeding season, we quantified reproductive success.
Methods

General methods

Breeding cavities are likely the key resource that limits reproduction in eastern bluebirds (Gowaty and Plissner 1998). Non-breeding birds (floaters) are present in suitable habitats, are sexually mature, and display breeding condition (Liu, pers. obs.). When breeding opportunities became available (the original box owners died or disappeared from a territory), these floaters frequently assumed the territory and bred with widowed birds.

This study was conducted from March to August 2005 and 2006 in two field sites, located in Southeastern Alabama, USA, with a total of 200 nest boxes. The distance between nest boxes was approximately 100 m. The habitat consisted mainly of pastures and hay fields surrounded by fragmented pine forests.

In this long-term research population, the birds were captured and banded in the early breeding season (March1-15), before the experiment began. At each field site, we also monitored all other unmanipulated territories. To capture birds, we lured pairs into mist nets by playing recordings of eastern bluebird calls. Each bird was individually marked with unique combination of three coloured bands and one U.S. Fish and Wildlife Service aluminum band. We measured body mass (accuracy = 0.1 g), and the length of tarsi, wings, and tails (accuracy = 0.5 mm). We used principle component analysis (PCA) to reduce our morphological measurements (tarsus,
wing, tail) into PC scores. Principal Component 1 explained 62.5% of the variation in morphological measurements. Body condition was calculated as the residuals of a regression of body mass on PC1 of body size (Jacob et al. 1996).

At the time of capture, we carefully plucked 8-10 rump (blue) and breast (chestnut) feathers. Feathers were placed in envelops and stored in a temperature and humidity controlled laboratory before analysis. We taped feathers onto black paper (Canson® Cat: #425 Stygian black) using an overlapping fashion to mimic how feathers naturally occur on the birds. We measured the plumage reflectance using an Ocean Optics S2000 spectrometer and deuterium tungsten halogen light source (range 250-880nm). We used a fiber optic cord equipped with a black rubber cap on the metal probe to exclude ambient light. The distance between the probe and feather was set to 5mm to create a measurement diameter of approximately 2mm. The probe was perpendicular to the feather surface. We took all colour measurements at the same setting and the same person (ML) processed all data. We generated reflectance data relative to a white standard (Labsphere, Inc.®) using OOIBase software (Ocean Optics®). We measured each feather sample five times and used the mean to generate colour scores for each male.

For chestnut coloration (Fig. 1), we summarized reflectance data by calculating two standard descriptors of reflectance spectra: brightness and chroma. Mean brightness was calculated as the mean summed reflectance (R<sub>300-700nm</sub>). Red chroma was calculated as the proportion of the total
reflectance ($R_{300-700nm}$) in the red part of the spectrum ($R_{575-700nm}$). For UV-blue colour (Fig. 1), we calculated three standard descriptors of the reflectance spectra: brightness, chroma, and hue. Mean brightness was calculated as the mean summed reflectance ($R_{300-700nm}$). UV chroma was calculated as the proportion of the total reflectance ($R_{300-700nm}$) in the UV part of the spectrum ($R_{300-400nm}$). Blue chroma was calculated as the proportion of the total reflectance ($R_{300-700nm}$) in the blue part of the spectrum ($R_{400-512nm}$). Hue was calculated as the wavelength at peak reflectance. Calculations taken from the same spectral curves are correlated in eastern bluebirds such that the most ornamented UV-blue males display brighter coloration, greater UV chroma, and hues with wavelengths shifted towards the shorter wavelengths (Siefferman et al. 2005). Moreover, the most ornamented chestnut males display darker coloration (lower brightness) and greater red chroma.

**Female removal**

In both years, we conducted the experiment within 3 weeks of the first egg date in the population (2005: 1 March – 15 March; 2006: 15 March - 1 April). For each removal, we choose two adjacent territories (within 200m of each other) such that each territory had a resident pair and the two pairs were synchronized in their breeding cycles (the females began laying eggs on the same date ($\pm$ 2 days)). Within 5 days of the date that the females of two adjacent pairs began laying eggs, we captured males and females of both pairs. On the morning of the capture, we released
males on their original territory within 30 min of capturing them and placed each female in captivity. Upon capture, the eggs and nest materials were collected from the nest boxes. In each year, the manipulated dyads of territories were geographically scattered at two study sites and occurred nearby or interspersed with other eastern bluebird territories where breeding pairs remained unmanipulated. Each day, we removed no more than two dyads of females.

We monitored the behaviour of the experimentally widowed males twice daily and determined the date in which they attracted a new female to their territory. After each male had secured a new mate, we monitored the breeding activities of the newly-formed pair throughout the breeding season. To reduce disturbance, replacement females were captured during the late incubation or early nestling stages.

Behavioral observations

We began behavioural observations of focal males within 12 hrs of the time of female removal. Each day following the removal, we monitored the behaviour of each focal male in the morning (60 min) to determine whether the male had attracted a replacement mate. During morning observations, we recorded male song rate (defined as number of songs per min) for approximately 30 min. The open grassland habitat and propensity of bluebirds to perch in the open, near their nest boxes, allowed for detection of new females. We determined that a new female had arrived if the
female was seen with the male for a minimum of 30 continuous mins and if the female was seen
with the focal male for two consecutive days. We digitally photographed pairs to confirm the
identity of the newly arrived female. After one of the males from each dyad had successfully
attracted a new female, we reduced our observation frequency to once every 2-3 days and
continued to monitor all the territories until the end of the breeding season. For all subsequent
nests, we recorded the date of the first laid egg of each brood, the clutch size, the brood size and the
fledging success.

After a female was removed from the original territory, widowed males sang and exhibited
display behaviours on or near the nest boxes (perched on boxes, repeatedly looked into nest holes,
waved wings, and carried nesting material). We recorded the song rates of males during morning
observations in 2006. Because some males attracted females quickly, and because song rate and
display behaviour were altered by the presence of the new females, we were able to record singing
behaviour of only 10 males. Song rate did not correlate with plumage colour (all $P > 0.1$), so we
eliminated song as a variable in subsequent analyses.

When newly arrived females were visible, we recorded behaviours indicative of mating
interest including when females foraged in the males’ territory, perched on boxes, repeatedly
looked into nest holes, followed (or was followed by) the male of the territory. Mate-guarding
behaviours are un-ambiguous in this species (pairs are usually observed within 20 m of each other
during courtship and egg laying) and we are confident that we could accurately determine when a male paired with a female (LS, personal observation). We used two measurements to describe the males’ success in attracting new females: 1) the latency period between female removal and attraction of new mate (days), (latency to attract, LA1), and 2) the time lapse between the date of the original female’s removal and the date that the replacement female commenced egg laying (latency to attract, LA2). We considered each dyad of widowed males to represent one trial. During the study, most of the males \( (N = 40) \) were only used once. However, 4 males were used in both 2005 and 2006 but all males were paired with new mates, occupied new territories, and were placed in dyads with unique males. In each trial, the male ‘won’ if he attracted a female to his territory before the other male in the dyad (‘winning’ males exhibited shorter LA1 than ‘losing’ males). In two cases, we missed the exact date that the winner attracted his female, but they were assigned by the first egg date of their mates.

Ethical note

This study was conducted with approval from IACUC (PRN: 2003-0448) at Auburn University. After capture, females were housed in outdoor flight cages of a permanent aviary at Auburn University (for details see Liu et al. 2007). Birds were given \textit{ad libitum} water, and live mealworms and crickets. Our aviary setting effectively reduced the cage stress; we bred a pair of
eastern bluebirds successfully in such an aviary cage in 2004. All birds were maintained in the aviary for approximately 4-6 weeks and then released to their original territories in the breeding season (mid-May) after the experiment. Because most bluebird pairs initiate second broods after mid-May, we expected that any females that could find a mate at this time had the opportunity to breed later in the season. We did observe that a released female bred successfully with a new male in June of 2006. Additionally, we recorded the return rate of the original (17.3%) versus replacement females (21.7%) in 2007. Captivity did not influence the likelihood that females would return to breed at the field site in the following year (Fisher’s exact test: \( P = 0.73 \)).

Statistical analyses

We used SPSS 11.5 (SPSS Inc. 2003) for all analyses. We used paired t-tests to compare male characteristics within each dyad. Additionally, we combined all data and used backwards and forward regression models to examine whether male characteristics predicted female arrival time, time to breed, and total seasonal reproductive success (number of offspring fledged). All data conformed to normality (Shapiro-Wilks’ tests), and parametric tests were used. Blue brightness differed by year, thus we standardized these data for year (mean = 0, SD = 1). All significance levels were two-tailed. Samples sizes vary between some variables because we failed to collect
feathers and morphological measurements of 3 males, and we did not collect plumage data from 10 females.

Results

Paired comparisons

Over the course of two breeding seasons, we conducted 24 trials of female removals (females were removed from 48 territories). Forty-one widowed males attracted a new mate after mate removal and all of these newly formed pairs bred successfully later in the breeding season. Four males disappeared from their territories after their original mates were removed. One female escaped from the aviary and reunited with her original mate. Of 19 successful trials, two trials yielded “tie” results (both males attracted replacement mates on the same day), and were eliminated from the paired comparisons. The median days to attract a replacement female (LA1) was 3 days (range: 0.5-58 days), and the median days from female removal until the replacement female laid her first egg (LA2) was 13 days (range: 7-63 days). LA1 and LA2 did not correlate \( r_s = 0.33, N = 31, P = 0.07 \).

Paired t-tests revealed that within dyads, there were no significant differences between the UV-blue coloration of the rumps of ‘winning’ and ‘losing’ males (Winners-losers; UV chroma: mean difference = -0.01 ± 0.03, \( t = 1.16, N = 15, P = 0.27 \); blue chroma: mean difference = -0.001
± 0.02, \( t = 0.20, N = 15, P = 0.85 \); brightness (\( z \)): mean difference = 0.34 ± 1.34, \( t = 0.98, N = 15, P = 0.34 \); hue: mean difference = 3.94 ± 19.76, \( t = 0.77, N = 15, P = 0.45 \), Table 1). Nor did we find a significant difference in chestnut coloration of the breast of ‘winning’ and ‘losing’ males (red chroma: mean difference = -0.01, \( t = 1.04, N = 13, P = 0.32 \); brightness: mean difference = 0.38, \( t = 0.67, N = 13, P = 0.52 \); Table 1). Further, morphological measurements did not differ significantly between ‘winning’ and ‘losing’ males (body size: mean difference = 0.19 ± 1.23, \( t = 0.56, N = 13, P = 0.59 \); body condition: mean difference = 10.64 ± 135.6, \( t = 0.28, N = 13, P = 0.78 \), Table 1). Over the course of the breeding seasons, the ‘winning’ and ‘losing’ males fledged a similar number of chicks (mean difference = 1.08 ± 3.90, \( t = 0.96, N = 12, P = 0.36 \), Table 1).

Because the manipulations that we conducted were technically difficult, our sample sizes were relatively small. Thus we used power analyses to determine whether our sample sizes were sufficient to find an effect if it existed. Previous studies that were laboratory based found that ≥ 80% of females preferred the males with the more-ornamented structurally-based plumage (Bennett et al. 1997, Hunt et al. 1999, Siitari et al. 2002). Given our sample sizes (\( N = 17 \)), we calculated that, if female bluebirds showed a similar preference to that of female bluetits and starlings, we had an 82% likelihood of finding an effect with our data set.

Male Comparisons
We treated each territory as an independent event and investigated the potential relationships between male characteristics, measures of mate attraction, and breeding success. We found no significant effects of year on either the time to attract a mate (t-test: LA1: \( t = 1.51, N = 15, 22, P > 0.1 \)) or time for the new mate to commence egg laying (LA2: \( t = 1.20, N = 15, 24, P > 0.1 \)).

All territories were manipulated in a relatively short period (three weeks), and we found no influence of removal date on the speed at which males attracted new mates (LA1, 2005: \( r_s = 0.37, N = 15, P = 0.17 \); 2006: \( r_s = 0.16, N = 22, P = 0.49 \)), nor the speed at which the new female laid her first eggs (LA2, 2005: \( r_s = -0.10, N = 15, P = 0.72 \); 2006: \( r_s = 0.1, N = 24, P = 0.65 \)). Additionally, because males of similar quality were more likely to occupy neighboring territories, we calculated the average colour of males within dyads to test whether the average dyad coloration predicted average time to attract new females. We found no significant relationship in any comparison (Table 2.). Finally, we used a mixed-model analysis with dyad ID as a random factor, we found no significant relationship between male coloration and female arrival latency (UV-blue rump brightness: \( F_{1,33} = 1.85, P = 0.18 \); UV chroma: \( F_{1,33} = 0.29, P = 0.60 \); blue chroma: \( F_{1,33} = 0.19, P = 0.67 \); hue: \( F_{1,33} = 0.19, P = 0.67 \). Chestnut breast brightness: \( F_{1,24} = 2.91, P = 0.10 \); red chroma: \( F_{1,25} = 0.88, P = 0.36 \).

We used backwards multiple regression models to determine the influence of male colour measurements and morphology on 1) the latency to attract (LA1), 2) the latency between female
removal and the day that the new female began laying eggs (LA2), and 3) the total offspring number of fledged. We found no significant influence of male coloration, body size, or body condition on latency to attract new females (Model included UV-blue brightness, chestnut brightness, and body size: $R^2 \text{ adj} = 0.05, F_{3, 21} = 1.41, P = 0.27$, Fig. 2), latency for new females to begin breeding (Model included chestnut red chroma, body condition, and body size: $R^2 \text{ adj} = 0.04, F_{3, 24} = 1.41, P = 0.27$, Fig. 2), or total number of offspring fledged in that season (Model included: UV-blue hue; chestnut brightness; chestnut red chroma: $R^2 \text{ adj} = 0.07, F_{3, 24} = 1.69, P = 0.20$, Fig. 3).

To further investigate these relationships, we used a series of forward multiple regression models to explore the potential influences of male characteristics on 1) the latency to attract (LA1) and 2) the latency between female removal and the day that the new female began laying eggs (LA2). The first model only included the six measurements of male plumage coloration as predictors variables and we found that coloration did not influence latency to attract new female (LA1, Full model: $R^2 \text{ adj} = 0.12, F_{5, 26} = 0.71, P = 0.62$) or the timing to commence laying eggs (LA2, Full model: $R^2 \text{ adj} = 0.12, F_{5, 28} = 0.74, P = 0.6$). In the next model, we only included male body size and body condition and, again, found no influence of male morphological characteristics on latency to attract a new female (LA1, Full model: $R^2 \text{ adj} = 0.10, F_{2, 25} = 1.42, P = 0.26$), or timing
to commence laying eggs (LA2, Full model: $R^2_{adj} = 0.10$, $F_{25}^2 = 1.58$, $P = 0.22$). Finally, a large model which included all measures of colour and morphology, found no influence on latency to attract a new female (LA1, full model: $R^2_{adj} = -0.05$, $F_{26}^5 = 0.71$, $P = 0.62$), or timing of new female to commence egg laying (LA2, full model: $R^2_{adj} = -0.04$, $F_{28}^5 = 0.74$, $P = 0.60$).

Finally, we also used discriminate function analyses (DFA) to identify any unique characteristics of ‘winning’ and ‘losing’ males. We combined all colour variables and morphological traits into these models. We found no significant difference between winning and losing group (Wilks’ lambda $F_{7,27} = 5.19$, $P = 0.64$). Based on previous studies of the same population (Siefferman and Hill 2005b), we selected three predictors (UV-blue brightness, UV chroma, and body size) for another DFA analysis. Again, we found that the overall model was not significant (Wilks’ lambda $F_{3,28} = 3.54$, $P = 0.32$) indicating that there was no significant differentiation in morphology or coloration of winning and losing males.

Assortative mating

Because pairs may mate assortatively for coloration, we tested whether the mated pairs’ plumage and body size characteristics were more similar than random using Pearson’s correlations. We found no significant relationships between the UV blue color (brightness $r = 0.02$, $N = 35$, $P = 0.91$; UV chroma $r = -0.03$, $N = 35$, $P = 0.85$; blue chroma $r = 0.02$, $N = 35$, $P = 0.93$;
hue \( r = 0.13, N = 35, P = 0.47 \), the chestnut color (brightness \( r = 0.02, N = 32, P = 0.93 \), red chroma \( r = 0.03, N = 32, P = 0.89 \)) or the body size \( r = 0.06, N = 38, P = 0.72 \) of the original female and her mate. Nor did we find any significant relationships between the UV-blue (UV-blue rump: brightness \( r = -0.13, N = 25, P = 0.55 \); UV chroma \( r = 0.09, N = 25, P = 0.67 \); blue chroma \( r = -0.16, N = 25, P = 0.43 \); hue \( r = 0.01, N = 25, P = 0.95 \)) the chestnut color (brightness \( r = 0.29, N = 24, P = 0.17 \); red chroma \( r = 0.02, N = 24, P = 0.93 \)) or body size \( r = 0.06, N = 25, P = 0.78 \) of the replacement female and her mate.

Discussion

Contrary to our hypothesis that female bluebirds prefer to pair with males that display the most-ornamented structural coloration, we found no evidence of mate choice based on plumage coloration. When we removed females and created experimentally widowed males, unmated females quickly settled in the opened territories. We found no evidence that females preferentially settled with the more-ornamented males. In comparisons of dyads of males that were carefully matched for territory quality and for timing of mate removal, the more-ornamented and larger males did not attract females more quickly compared to the less-ornamented and smaller males. Although we did not find evidence for a female preference, it is possible that a much larger sample size might have shown a trend.
The observation from our field manipulation that female eastern bluebirds do not use male coloration as a criterion in mate choice is consistent with aviary-based mate choice trials in which female eastern bluebirds chose males without regard to either manipulated UV-blue plumage coloration or natural variation in UV-blue plumage coloration (Liu et al. 2006). The lack of influence of male body size, body condition, or chestnut coloration on female mate-choice decisions is also consistent with results of the aviary-based trials (Liu et al. 2007, M. Liu, L. Siefferman, G. Hill, unpublished data).

Our experimental design differs from most approaches to mate choice in three important ways. First, unlike most field-based tests of mate choice, we matched pairs of widowed males on adjacent territories. This design was employed to control for territory quality as we assume that males, in the relatively homogenous grassland habitat of eastern bluebirds, with adjacent territories will have territories of similar quality. Second, unlike most field-based research of mate choice, we removed original females thus eliminating any influence of the pair’s prior breeding experience. Third, unlike most laboratory-based experiments of mate choice, our design is unique because females were free to interact with males, and to reproduce, and we quantified subsequent reproductive success.

Past research with bluebirds demonstrates that males that display more exaggerated blue and chestnut coloration pair earlier and feed offspring more often (Siefferman and Hill 2003),
suggesting that female choice of more-elaborately ornamented males would be adaptive. Further, (Siefferman and Hill 2005b) found that UV-blue structural coloration is a good indicator of the ability of males to secure limited nest boxes, suggesting that males with more elaborate UV-blue coloration have advantages in competitive interactions for breeding sites. In this population, however, older male birds also tend to display brighter UV-blue coloration and duller chestnut coloration compared to younger males (Siefferman et al. 2005). The positive correlations between plumage coloration and reproductive success in eastern bluebirds could be a consequence of older pairs experiencing greater reproductive success because of increased mate familiarity and reproductive experience (Black 1996). Indeed, in this population, pairs mate assortatively by age and older females tend to breed earlier and experience greater seasonal reproductive success (Siefferman & Hill 2005c).

The newly-formed pairings that resulted from our experiment might have disentangled the past interrelationships between length of pair bond, age of birds, male plumage coloration, and reproductive success (Siefferman & Hill 2003). Perhaps females prefer to nest with males with which they have had prior nesting or social experience and those males tend to express greater UV-blue coloration because they tend to be older. The lack of effect of colour on timing of attraction of replacement mates suggests that females are not actively seeking more colorful males.
Several studies have found that pair duration and previous breeding experience are important determinants of reproductive success in socially monogamy species (Ligon 1999) and past pair bonds are likely important in this population of bluebirds. The majority of the males that were experimentally widowed successfully attracted females. In addition to experimentally manipulated pairs, we banded and monitored the nesting attempts of all bluebirds that bred on our study site (80 pairs) and found that no females left their original mates to pair with experimentally widowed males. Moreover, all replacement females arrived on our field site without bands, suggesting that those females came from the floating pool of birds.

Our data are consistent with research suggesting that the cost of leaving a mate is generally greater than the benefits gained from pairing with a higher-quality mate (Ligon 1999; but see Otter and Ratcliffe 1996). In general, females that left their mates experience the costs of delayed breeding (Black et al. 1996). Because species that nest exclusively in secondary cavities have historically experienced very limited nest sites, females that chose to leave their original mates face the risk of forsaking all breeding opportunities in that breeding season (Gowaty 1981). In our population, most pairs breed from March to August and are able to produce two or three successful broods. Pairs that initiate egg laying earlier in the season experience greater seasonal reproductive success (Siefferman & Hill 2005c). Indeed, in swans, mate familiarity has been shown to reduce the costs of courtship and facilitate earlier breeding (Rees et al. 1996).
Our experimental design helped us disentangle the confounding effects of coloration, prior pairing history, and reproductive success that may naturally occur in eastern bluebirds. We found no evidence that female eastern bluebirds seek out more colourful males as mates. We also found no evidence that pairs mate assortatively for coloration or body size, again this is consistent with field correlational evidence (Siefferman & Hill 2005c). It is possible, however, that female bluebirds may not all display similar mate choice preferences. Several researchers have demonstrated that female mating preference might vary depending on environment, prior experience, and body condition (Qvarnstrom 2001). It is also possible that coloration functions in male-male interactions and signals male condition. Further, it is possible that females use male plumage coloration to choose extra-pair mates, as has been found in the sister species, mountain bluebirds (Sialia currucoides; Balenger et al. 2009). Future research should investigate individual variation in mate preferences and the signaling function of male coloration and genetic quality in eastern bluebirds (Mays & Hill 2004; Mays et al. 2008).

Acknowledgements

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N. Hart and two anonymous reviewers for improvements to the manuscript. The National Science Foundation grants to GEH (IBN 0235778, and DEB 0077804), National Institute of Health/National Science Foundation program in the Ecology of Infectious Diseases R01-AI49724 to GEH funded this research. This research was conducted according to an animal use permit from Auburn University, banding permits to GEH.
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Table 2.1 Comparisons of characteristics of all males that gained a mate first (won) or second (lost), paired-t test.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Outcome</th>
<th>Mean</th>
<th>SD</th>
<th>N</th>
<th>t</th>
<th>P</th>
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<tbody>
<tr>
<td>Tarsus (mm)</td>
<td>Won</td>
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<td>16</td>
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<td>0.99</td>
</tr>
<tr>
<td></td>
<td>Lost</td>
<td>20.48</td>
<td>0.73</td>
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<td></td>
</tr>
<tr>
<td>Wing (mm)</td>
<td>Won</td>
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<td>2.27</td>
<td>16</td>
<td>0.25</td>
<td>0.81</td>
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<tr>
<td></td>
<td>Lost</td>
<td>100.78</td>
<td>2.89</td>
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</tr>
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<td>Tail (mm)</td>
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<td>16</td>
<td>1.83</td>
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<td>Lost</td>
<td>62.91</td>
<td>2.58</td>
<td>16</td>
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<td></td>
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<tr>
<td>Mass (g)</td>
<td>Won</td>
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<td>15</td>
<td>0.07</td>
<td>0.94</td>
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<td>Lost</td>
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</tr>
<tr>
<td>Body Condition (z)</td>
<td>Won</td>
<td>1.45</td>
<td>0.07</td>
<td>15</td>
<td>0.28</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>Lost</td>
<td>1.45</td>
<td>0.11</td>
<td>15</td>
<td></td>
<td></td>
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<tr>
<td>Age (yr)</td>
<td>Won</td>
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<td>Lost</td>
<td>1.67</td>
<td>0.49</td>
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<tr>
<td>Rump Brightness (z)</td>
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<td>1.01</td>
<td>15</td>
<td>0.98</td>
<td>0.34</td>
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<tr>
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<td>Rump UV Chroma (z)</td>
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<td>0.02</td>
<td>15</td>
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<td>0.02</td>
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<td>Rump Blue Chroma (z)</td>
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<td>0.01</td>
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<td>0.01</td>
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<td>Rump Hue (z)</td>
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<td>12.37</td>
<td>15</td>
<td>0.77</td>
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<td>Lost</td>
<td>413.93</td>
<td>9.12</td>
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<tr>
<td>Breast Brightness (z)</td>
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<td>1.20</td>
<td>13</td>
<td>0.67</td>
<td>0.52</td>
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<td>Lost</td>
<td>9.74</td>
<td>1.68</td>
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<td>Breast Red Chroma (z)</td>
<td>Won</td>
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<td>0.03</td>
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<td>-1.03</td>
<td>0.32</td>
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<tr>
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<td>0.02</td>
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</tr>
<tr>
<td>LA 1 (days)</td>
<td>Won</td>
<td>3.00</td>
<td>2.56</td>
<td>11</td>
<td>-3.23*</td>
<td>0.001</td>
</tr>
<tr>
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<td>11.36</td>
<td>15.98</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA 2 (days)</td>
<td>Won</td>
<td>12.77</td>
<td>3.32</td>
<td>13</td>
<td>-2.03*</td>
<td>0.04</td>
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<tr>
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<td>17.58</td>
<td>13</td>
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<tr>
<td>Breeding success (young)</td>
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<td>2.08</td>
<td>12</td>
<td>0.96</td>
<td>0.36</td>
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<tr>
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<td>Lost</td>
<td>5.75</td>
<td>2.90</td>
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</table>
Table 2.2 Correlations between the mean plumage coloration of males in each dyad and the mean latency to attracted a mate for each dyad (LA1).

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<thead>
<tr>
<th>Trait</th>
<th>r_s</th>
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<th>P</th>
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<td>Mean Rump Brightness (z)</td>
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<td>16</td>
<td>0.09</td>
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<tr>
<td>Mean Rump UV Chroma (%)</td>
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<td>16</td>
<td>0.63</td>
</tr>
<tr>
<td>Mean Rump Blue Chroma (%)</td>
<td>0.27</td>
<td>16</td>
<td>0.31</td>
</tr>
<tr>
<td>Mean Breast Brightness (%)</td>
<td>-0.34</td>
<td>15</td>
<td>0.22</td>
</tr>
<tr>
<td>Mean Breast Red Chroma (%)</td>
<td>0.24</td>
<td>15</td>
<td>0.39</td>
</tr>
</tbody>
</table>
Figure 2.1

Mean (±1SE) reflectance spectra of the UV-blue of rumps (thick line) and the chestnut coloration of breasts (thin line) male eastern bluebirds (n=16).
Figure 2.2

Relationship between brightness of UV-blue rump coloration of the male eastern bluebirds and 1) time to acquire a new mate (open circles), and 2) time to begin breeding with new mate (filled circles). Colour data are standardized for year.
Figure 2.3

Relationship between brightness of UV-blue rump coloration of the male and the seasonal reproductive success (total number of nestlings fledged per year) of eastern bluebirds. Colour data are standardized for year.
Chapter 3. Ornamentation versus Genetic Compatibility as Criteria in Mate Choice
Introduction

Females achieve greater reproductive success by choosing high-quality mates who provide either good genes or material resources. Trivers (1972) suggested that an important measure of the genetic quality of a potential mate is how complementary is his genotype to that of the female. More complementary genotypes produce more heterozygous offspring, which may exhibit higher immune function (Hawley et al. 2005), better survival (Foerster et al. 2003) or display greater ornamentation (Marshall et al. 2003). To produce more heterozygous offspring, females should choose a genetically dissimilar mate, and under such self-referential mate choice, the ideal mate for each female will be different. This hypothesis for mate choice runs counter to traditional ‘good genes’ models of sexual selection (Mays and Hill 2004), in which females are expected to choose males with universally good genes for traits such as disease resistance. Under the good genes model, all females should choose the same, most highly ornamented males.

To date, despite accumulating evidence for the importance of genetic compatibility in sexual selection, no study has experimentally tested female preferences for ornamentation versus genetic compatibility in a wild population of vertebrates. Here, we tested whether female eastern bluebirds prefer more genetically compatible males or males with the brightest feather coloration.

Methods

General methods

This study was conducted from March to August 2005 and 2006 in two field sites, located in Southeastern Alabama, USA with a total of 200 nest boxes. The distance between nest boxes was approximately 100 m. The habitat consisted mainly of pastures and hay fields surrounded by fragmented pine forests. In this long-term research population, the birds were captured and banded
in the early breeding season (March 1 - March 15), before the experiment began. At each field site, we also monitored all other unmanipulated territories. Each bird was individually marked with unique combination of three coloured bands and one U.S. Fish and Wildlife Service aluminum band. At the time of capture, we carefully plucked 8-10 rump (blue) and breast (chestnut) feathers. Feathers were placed in envelopes and stored in a temperature and humidity controlled laboratory before analysis. For further color analysis see Siefferman et al. (2005).

Female removal

In both years, the experiment was conducted within 3 weeks of the first egg date in the population (2005: 1 March – 15 March; 2006: 15 March - 1 April). For each removal, we choose two adjacent territories (within 200m of each other) such that each territory had a resident pair and the two pairs were synchronized in their breeding cycles (the females began laying eggs on the same date (+ 2 days)). Within 5 days of the date that the females of two adjacent pairs began laying eggs, we captured males and females of both pairs. On the morning of the capture, we released males on their original territory within 30 min of capturing them and placed each female in captivity. Upon capture, the eggs and nest materials were collected from the nest boxes. In each year, the manipulated dyads of territories were geographically scattered at two study sites and occurred nearby or interspersed with other eastern bluebird territories where breeding pairs remained unmanipulated. Each day, we removed no more than two dyads of females.

We monitored the behaviour of the experimentally widowed males twice daily and determined the date in which they attracted a new female to their territory. After each male had secured a new mate, we monitored the breeding activities of the newly-formed pair throughout the
breeding season. To reduce disturbance, replacement females were captured during the late incubation or early nestling stages.

Behavioral observations

We began behavioral observations of focal males within 12 hrs of the time of female removal. Each day following the removal, we monitored the behaviour of each focal male in the morning (60 mins) to determine whether the male had attracted a replacement mate. During morning observations, we recorded male song rate (defined as number of songs per min) for approximately 30 mins. The open grassland habitat and propensity of bluebirds to perch in the open, near their nest boxes, allowed for detection of new females. We determined that a new female had arrived if the female was seen with the male for a minimum of 30 continuous mins and if the female was seen with the focal male for two consecutive days. We digitally photographed pairs to confirm the identity of the newly arrived female. After one of the males from each dyad had successful attracted a new female, we reduced our observation frequency to once every 2-3 days and continued to monitor all the territories until the end of the breeding season. For all subsequent nests, we recorded the date of the first laid egg of each brood, the clutch size, the brood size and the fledging success.

When newly arrived females were visible, we recorded behaviours indicative of mating interest including when females foraged in the males’ territory, perched on boxes, repeatedly looked into nest holes, followed (or was followed by) the male of the territory. Mate-guarding behaviours are un-ambiguous in this species (pairs are usually observed within 20 m of each other during courtship and egg laying (LS, pers. obs.)) and we are confident that we could accurately determine when a male paired
with a female.

DNA genotyping

For microsatellite genotyping, we used total 10 microsatellite loci (5 tetranucleotide loci: SIALIA11, SIALIA22, SIALIA27, SIALIA36, SIALIA37 (Faircloth et al. 2006) and 5 dinucleotide loci: EABL129, MOBL47, MOBL49, MOBL53, MOBL 86b (Ballinger et al. 2009). A PTC-100 thermocycler (MJ Research) was used to amplify the microsatellites with the following program: 94° C for 10 min, 35 cycles of 30 s at 94° C, 30 s at 50° C, 30 s at 72° C and final elongation at 72° C for 10 min. Each 10 µL polymerase chain reaction (PCR) contained ≈250 ng of total DNA, 5 µl of MasterMix (Sigma-Aldrich), 0.25 µM of forward primer, 0.25 µM of fluorescent M13 primer (Sigma-Proligo) and 0.5 µM of backward primer (Invitrogen Life Technologies). The PCR product was analyzed following the standard protocol for the CEQ 8000 Genetic Analysis System (Beckman Coulter).

We calculated an index of mate compatibility (C) derived from the $d^2$ statistic, for each female and chosen mate (Coulson et al. 1998). C is the mean square difference of all four pair-wise comparisons of a female’s allele sizes to those of her mate, average across loci

$$C = \frac{1}{n} \sum_{i=1}^{n} [(a_{M1} - a_{F1})^2 + (a_{M2} - a_{F1})^2 + (a_{M1} - a_{F2})^2 + (a_{M2} - a_{F2})^2]$$

where $a_{M1}$ and $a_{M2}$ are the two alleles of the male at locus i, $a_{F1}$ and $a_{F2}$ are the two female alleles at the same locus, and n is the total number of loci (Smith et. al 2005). We obtained 2 different C scores from each dyad, first C score was calculated from the first arrived female and the
male she chosen. Second C score was calculated from the same female and the other male she did not choose.

Results

In the field, we presented choosing females with dyads of males by widowing males and allowing replacement females to choose between them. We controlled for the influence of territory quality on female choice by widowing dyads of males with adjacent territories. Females preferred the most genetically compatible ($X^2 (1,11) = 4.46, P= 0.035$) not the most-ornamented potential mate ($X^2 (1,16) = 1, P= 0.32$) (Figure 1). Additionally, our data show that females also tended to pair with the more heterozygous mates ($X^2 (1,11) = 4.46, P= 0.035$). To our knowledge, this is the first experimental evidence in birds that female use genetic criteria in choosing social mates.

Discussion

Our data also suggest that females are capable of using different mate choice criteria under difference scenarios. In this population, male coloration serves as a signal of male resources holding potential (Siefferman and Hill 2005b), and earlier in the season brightly colored males pair faster than drably colored males. Moreover, females to brighter males experience greater reproductive success(Siefferman and Hill 2003). In this study, however, we experimentally controlled variation in territory quality and found that females use genetic criteria to choose mates. A similar hierarchical decision making strategy has been found in lab mice (Roberts and Gosling 2003). When variation in signals of social dominance is relatively low and genetic variation among mates is high, females switched preferring the dominant male to preferring the more genetic compatible male. To date, few avian studies have tested whether females use genetic compatibility
criteria to choose mates and results have been equivocal (Mays et al. 2008). Because environmental resources such as territory quality also influence reproductive success, it may be difficult to detect female preferences for male traits. Context-dependent female mating strategies may provide a potential route to conciliate the incongruence of mate choice for genetic compatibility in birds (Qvarnstrom 2001; Roberts and Gosling 2003; Oh and Badyaev 2006; Mays et al. 2008).

Acknowledgements

We are grateful to B. Rolek, and M. Rios for excellent help with data collection, and R. Montgomerie for the use of his spectral processing program (ColouR v1.7). We thank S. Balenger, W. Hood, R. Ligon for improvements to the manuscript. The National Science Foundation grants to GEH (IBN 0235778, and DEB 0077804), National Institute of Health/ National Science Foundation program in the Ecology of Infectious Diseases R01-AI49724 to GEH funded this research. This research was conducted according to an animal use permit from Auburn University, banding permits to GEH.
References


Figure 3.1 (a) Female’s mating preference based on male’s rump brightness, $X^2 (1,16) = 1$, $P = 0.32$. Ten out of sixteen trials female chose to mate with brighter males (b) Female mating preference based on male’s genetic compatibility, $X^2 (1,11) = 4.46$, $P = 0.035$. Nine out of 11 trials female chose to mate with genetically more compatible males.
Chapter 4. Female Reproductive Success in Relation to Male Ornamentation and Genetic Compatibility of Pairs
Abstract

Male eastern bluebirds (Sialia sialis) vary in the elaborateness of their structurally-based blue feather color and in their genetic compatibility relative to prospective mates. Here, we measure the benefits to females of pairing with highly-ornamented males versus males that are more genetically compatible. This species is socially monogamous; both parents care for offspring and pairs produce multiple clutches during a breeding season. We found that females mated to brighter males fledged more offspring annually and that both male coloration and genetic compatibility of pairs predicted offspring quality. The relative importance of genetic compatibility versus mate ornamentation on nestling quality, however, varied with season. For first clutches, early in the breeding season, genetically more-compatible pairs produced heavier nestlings. On the other hand, for second clutches, later in the season, brighter males produced offspring that were heavier and had longer wings at fledging. We found no relationships, however, between male heterozygosity and either coloration or reproductive success. Our data suggest that females receive benefits both from mating with genetically more-compatible males and mates that are more colorful, however, environmental circumstances influence the relative benefits of mate selection for ornamentation versus genetic compatibility.

Key Words: genetic compatibility, good genes indicator models, ornamentation, UV-blue structural coloration, reproductive success, Sialia Sialis
Introduction

Directional selection from female mate preferences for conspicuous male ornaments is one of the central mechanisms explaining exaggerated sexual ornaments (Andersson 1994; Darwin 1871). In the past three decades, numerous empirical studies have demonstrated evidence for female mate choice for highly-ornamented male birds (Hill 2006). It is argued that if ornamentation is an honest and reliable signal of male resource-holding ability or male genetic quality, females could either gain direct reproductive benefits or indirect reproductive benefits from mating with the most highly-ornamented males in the population (review in Griffith and Pyrak 2006). In particular, the good genes hypothesis emphasizes that genes for increased fitness could be identified by assessing male ornamentation (Hamilton and Zuk 1982; Jager et al. 2007; von Schantz et al. 1996), and that females that select highly-ornamented males would produce higher-quality offspring. A key assumption of this model is that “good genes” yield additive genetic benefits (Fisher 1930; Grafen 1990; Lande 1981).

Additive genetic benefits, however, are not the only source of genetic benefits to be gain through mate choice (Brown 1997; Hunt et al. 2004; Trivers 1972; Zeh and Zeh 2003). Non-additive genetic benefits can result if females chose males with genotypes that are compatible to their own, and thus produce heterozygous offspring (i.e. heterozygous advantage; (Tregenza and Wedell 2000). This genetic compatibility model of sexual selection hypothesizes that offspring fitness is not simply the sum of better and worse genes but is instead a result of the interactive effects of male and female genes. In particular, more heterozygous gene combinations may produce the most-fit genotypes. The model predicts that females should choose mates having a genotype that best complements her own genotype in order to produce adaptive and effective gene combinations in offspring(Mays and Hill 2004; Tregenza and Wedell 2000).
Mate choice for highly-ornamented males and mate choice for males that are genetically more compatible are fundamentally different (Mays et al. 2008; Mays and Hill 2004; Neff and Pitcher 2005). By comparing the reproductive consequences of pairing for genetic compatibility versus ornamentation in a wild population, the relative importance of the two mate choice criteria can be evaluated.

Eastern bluebirds (*Sialia Sialis*) are territorial, socially monogamous, and sexually dichromatic passerines. Male eastern bluebirds display bright UV-blue structural coloration on the plumage of their backs, heads, wings, and tails and chestnut phaeomelanin coloration on their breasts while females are overall duller in coloration. There is evidence from our experimental population that sexual selection may be acting on male plumage coloration. Males that express brighter UV-blue plumage and darker melanin breast color pair earlier, feed chicks more often, and experience higher reproductive success (Siefferman and Hill 2003). Brighter males are also more likely to acquire limited nesting sites suggesting color may influence male-male contests for breeding territories (Siefferman and Hill 2005c). Moreover, experimental manipulations of paternal investment indicate that this blue color is a condition-dependent trait in bluebirds (Siefferman and Hill 2005b), and thus could reliably signal male quality.

Here, we test two competing hypotheses of mate selection: the genetic compatibility and good gene hypotheses. First, we test whether female reproductive parameters are correlated with male ornamentation, male heterozygosity, and genetic compatibility of pairs of breeding birds. Second, we test whether male coloration signals heterozygosity.

Methods

General Methods
We monitored the breeding parameters of a box-nesting population of eastern bluebirds throughout the entire breeding seasons (March - August) of 2004 and 2005 in Auburn, AL. This long-term research population has been banded since 1999 (standard banding and measuring procedures can be found in Siefferman and Hill 2003). All breeding adults were captured during the early (March-April) breeding season. Upon capture, we punctured a brachial vein, collected 10ul of blood, and stored it at -20°C. We carefully plucked eight blue feathers from the rump of each bird, and layered and taped the feathers to black paper (Stygian Black, Canson # 425) for measurements using reflectance spectrometry.

We measured three different breeding parameters to represent reproductive success. First, we calculated the total number of offspring fledged during the entire breeding season for each focal female, which we refer to as total reproductive success. Because we protect nest boxes from predators (< 10% of nests are depredated), total reproductive success reflects true breeding potential. Second, we calculated hatching failure per nesting attempt as the number of unhatched eggs / clutch size. Third, as an estimate of nestling quality, we measured nestling mass at ages 5, 10, and 15 days and feather length at 15 days post hatch (hatching day = day1).

Plumage color analysis

We recorded spectral data from rump feathers of males with an Ocean Optics S2000 spectrometer (range 250-880nm: Dunedin, Florida, USA) using a micro fiber-optic probe held at 90° to the feather surface (see detailed methods in Siefferman and Hill 2003). We used a spectral processing program (ColorR v1.7, R. Montgomerie, Queens University, Ontario CA) to summarize color data using three standard descriptors of reflectance spectra: brightness, UV chroma, and hue. We define brightness, or total amount of light reflected by the feather, as the
summed reflectance from 300-700 nm. UV-blue chroma, a measure of spectral purity, was calculated as the ratio of the UV-blue reflectance (300-500nm) to the total reflectance (300-700 nm). Hue, the principal wavelength reflected by the feather, was calculated as the wavelength with the greatest reflectance.

Genotyping

We extracted blood samples using standard phenol-chloroform-isoamyl DNA extraction procedures (Sambrook and Russell, 2001). We genotyped five dinucleotide microsatellite loci (EABL129, MOBL47, MOBL49, MOBL53, MOBL 87b; primers published in (Balenger et al., 2009), and five tetranucleotide microsatellite loci (SIALIA11, SIALIA22, SIALIA27, SIALIA36, SIALIA37; primers published in Faircloth et al. 2006). Each 10 μL polymerase chain reaction (PCR) contained ≈250 ng of genomic DNA, 5μl of Fideli Taq™ PCR Master Mix (USB Corporation), 0.25μM of forward primer, 0.25 μM of WellRED (Beckman Coulter) fluorescent-labeled M13 primer (Sigma-Proligo), and 0.5 μM of reverse primer (Invitrogen Life Technologies). The forward primer was an M13-tailed primer(Balenger et al., 2009). Amplification were carried out using a PTC-100 thermocycler (MJ Research) with the following program: 94° C for 4 min, 30 cycles of 1 min at 94° C, 1 min at 50° C, 2 min at 72° C and final elongation at 72° C for 10 min. We separated and sized PCR products using a CEQ™ 8000 Genetic Analysis System Version 10 (Beckman Coulter Inc., Fullerton, CA) and scored alleles by eye based on rounding fragment size calls to the nearest base. We calculated allele frequency and heterozygosity using the Cervus 3.0 software. Based on 300 individual genotypes, we found that two out of ten loci did not conform to Hardy-Weinberg Equilibrium (Table 1).
Genetic compatibility index

We calculated an index of mate compatibility (C) derived from the d^2 statistic, for each paired female and male (Coulson et al., 1998). C is the mean square difference of all four pair-wise comparisons of the size of each female’s allele to those of her mate, averaged across all loci

\[ C = \frac{1}{n} \sum^n [(a_{iM1} - a_{iF1})^2 + (a_{iM2} - a_{iF1})^2 + (a_{iM1} - a_{iF2})^2 + (a_{iM2} - a_{iF2})^2] \]

where aiM1 and aiM2 are the two alleles of the male at locus i, aiF1 and aiF2 are the two female alleles at the same locus, and n is the total number of loci (Smith et al. 2005). This genetic compatibility index yields the measured potential combinations and allele differences of the offspring.

Male Heterozygosity index

We measured the internal genetic diversity of males by calculating the unit difference between two alleles at each locus, and summed the units across 10 loci

\[ \text{Mean } d^2 = \frac{1}{n} \sum^n \left( \frac{(a_i - a_j)^2}{u_i} \right) \]

where ai and aj refer to lengths of each allele at a locus, u_i refer to the nucleotide number of the locus unit, averaged over n typed loci (Coulson et al. 1998).

Statistical analyses

We tested for year effects on color parameters, and because color varied with year (T-test: Brightness: t = -2.44, p = 0.02; UV chroma: t = -5.24, p < 0.01; Hue: t = 3.12, p < 0.01), we standardized the data for year (z scores, mean = 0, standard deviation = 1) and combined both years of data. No breeding parameters or genetic parameters varied significantly with year.
To identify whether male color, male heterozygosity, and pair genetic compatibility influenced total reproductive success, we used backwards-stepwise linear regressions. To identify whether male color, male heterozygosity, and pair genetic compatibility influenced nestling growth parameters or hatching success per brood, we performed linear mixed effects models where female identity was included as the random factor and early versus late brood was included as an additional fixed factor. For each analysis, we included all explanatory variables and then removed them sequentially if they did not contribute significantly to the model (p>0.10).

The majority of pairs reared two successful broods within the breeding season; however, two females from 2004 and three females in 2005 had two different mates during the breeding season, thus we eliminated those pairs from further analyses. We tested normality using Shapiro-Wilk tests. We analyzed data using SPSS v. 17 for Windows; all tests were two-tailed.

Results

Influence of male color, heterozygosity, and genetic compatibility on annual reproductive success

Total reproductive success per female was $5.59 \pm 2.26$ (average ±SD) and ranged from 0-10 nestlings. In the final backward regression model, male brightness, hue, and heterozygosity were retained ($R^2 = 0.26$, $F_{3,39} = 4.50$, $p = 0.008$). Females mated to brighter males brightness (Beta = 0.58, $p = 0.05$), males with longer wavelength hues (Beta = 0.72, $p = 0.03$) and lower heterozygosity (Beta = -0.12, $p = 0.06$) fledged more offspring. In addition, we use simple correlations to examine the relationships between annual reproductive success and male’s characteristics. We found brightness was the single positive predictor ($r = 0.31$, $N = 44$, $p = 0.04$); neither genetic compatibility index($r = -0.07$, $N = 48$, $p = 0.62$), nor male’s heterozygosity ($r = -0.12$, $N = 48$, $p = 0.4$) predicted the annual reproductive success (Fig. 1a,b,c).
Influence of male color, heterozygosity, and genetic compatibility on hatching failure

Overall, 20% ± 19% (± SD; Range = 0% - 80%) of the eggs in the population failed to hatch. We found no significant interactions between male characteristics and season on percent hatching failure (Brightness: F2,39 = 0.46, p = 0.63; UV-blue Chroma: F2,39 = 0.64, p = 0.53; Hue: F2,39 = 0.03, p = 0.98), and no effect of season on hatching success (t = 0.03, N = 45, p = 0.98). We also found no significant relationships between percent hatching failure and 1) parameters of male color (Brightness: Rs = -0.11, N = 42, p = 0.49; UV-blue Chroma: Rs =-0.27, N=42, p=0.08; Hue: Rs = 0.02, N = 42, p = 0.88), 2) male heterozygosity (Rs = -0.02, N = 44, p = 0.9), or 3) genetic compatibility of pairs (Rs = 0.13, N = 44, p = 0.42). However, seasonal reproductive success was negatively correlated to the hatching failure (Rs = -0.35, N = 45, p = 0.02)

Influence of male color, heterozygosity, and genetic compatibility on nestling size

Nestling size was influenced by a significant interaction between season and male color (F1,62.6 = 7.44, p = 0.001). Therefore, we separated early and late season data to investigate the effects of male coloration and pair compatibility on nestling parameters (Table 2). Only male brightness and pair compatibility were retained in the final models. In the early season, females that were mated with genetically more-compatible males reared heavier nestlings at age 5, and 10 days (Table 2; Fig. 2a,b) but these relationships did not occur in the late season (Table 2). However, in the late season, females mated with brighter males reared heavier offspring at ages 5 and 10 days and nestlings with longer wings at age 15 days (Table 2; Fig. 3a,b). Nestling mass at age 15 days was not related to either male color or mate compatibility (Table 2).

Male heterozygosity and color
Male heterozygosity was not significantly related to any measure of UV-blue structural coloration (Brightness: $r = -0.12$, $N = 43$, $p = 0.46$; UV-blue Chroma: $r = -0.06$, $N = 43$, $p = 0.70$; Hue: $r = 0.00$, $N = 43$, $p = 0.99$).

Discussion

We found a significant effect of male color but no effect of pair compatibility on total reproductive success; females mated to brighter males produced more offspring. Both male ornamentation and compatibility had significant effect on offspring quality, but seasonality determine which male characteristic had the largest effect. In the early season, females paired with genetically more-compatible males reared heavier nestlings. In the late season, females paired with brighter males produced heavier nestlings and the nestlings that fledged with longer wings. Our study is the first to assess the relative pay off of female mate choice for male ornamentation versus genetic compatibility. Our observations suggest that the relative benefits of each type of mate choice to female reproductive success likely vary with environmental conditions.

Because we found significant relationships between reproductive success and both male color and mate compatibility, it is logical to wonder whether females choose mates by assessing male color or via some mechanism of assessing mate compatibility. In previous studies of this population, we experimentally demonstrated that female eastern bluebirds do not choose mates based on plumage coloration (Liu et al. 2007; Liu et al. 2009). Rather, females show preferences for mates with genetically more-compatible genotypes and males with more heterozygous genotypes (Liu et al. Chapter 3). Past research also suggests that brighter males are higher quality mates; brighter males feed nestlings more often and fledge more offspring (Siefferman & Hill 2003). In the current study, we found similar trends between male coloration and female
reproductive success. If females benefit from pairing with brighter males, why don’t they choose brighter males as partners? And if females do not select the brightly color males, what selection pressure maintains the traits in males? Female mate choice is only one mode by which sexual selection can work. Differential access to mates can also proceed through intra-sexual selection if ornaments signal resource-holding potential (Andersson 1994). An experimental manipulation of nest site availability indicates that plumage color in male eastern bluebirds is associated with competitive ability, suggesting that color may signal male resource holding ability (Siefferman and Hill 2005a). If female chose territories rather than plumage color, females paired with brighter males will experience higher reproductive success because those males defend higher-quality territories.

The relationship between nestling quality and paternal coloration appear to be complex and influenced by seasonality. Females mated with genetically more-compatible males produced nestlings of higher quality early in the early season, which explains why females selectively mate with genetically more-compatible males (Liu et al. Chapter 3). What mechanism is responsible for a seasonal shift in how male characteristics influence offspring quality? First, reproductive success may be a consequence of differential allocation of resources (egg quality, maternal care of offspring) by females in response to mate characteristics (Burley 1988). Females that pair with more compatible males may invest more resources in the first brood because the first brood is the most valuable in multiple-brooded species (Robinson 2005). To properly test the differential allocation hypothesis, researchers must manipulate the female’s perception of male quality and measure relative female investment (Sheldon 2000).

Additionally, the positive relationships between male plumage color and nestling growth could be a consequence of increasing value of high quality territories late in the nesting season. In
the late season, territory quality may become more important to parental reproductive success and override the positive effects of genetic quality. In this population, food supply shifts with season; as the breeding season progresses parents feed offspring larger insects and deliver food less often (Siefferman and Hill 2007). We suspect that this shift in environmental resources may increase the benefits of breeding in high quality territories that are usually defended by brighter males. Although the relationships between nestling quality, mate compatibility, and paternal coloration varied with season, brighter males fledged a greater number of offspring throughout the breeding season. Eastern bluebirds usually fledge two to three broods in a breeding season and brighter pairs usually start breeding earlier in the year and breed longer which allows them to maximize the number of offspring fledged (Siefferman and Hill 2005a). The relationship between color and total reproductive success may be partially a consequence of age; older males tend to be brighter (Siefferman et al. 2005); pairs mate assortatively for age; and older females commence egg laying earlier in the season (Siefferman and Hill 2005a). As in other species (Fowler 1995), the ability of older or more colorful birds to retain or gain higher quality territories may drive these relationships.

We also failed to detect any effect of male characteristics on hatching failure in this study. These results are surprising because increased relatedness of the breeding pairs has been implicated in hatching failure of other bird species (Hansson et al. 2004; Spottiswoode and Moller 2004). Perhaps we found no relationship between genetic compatibility of pairs and hatching success because our study population is not inbred. We measure a yearly influx of ~30% of breeding birds arriving each year from outside the study area. To our knowledge, none of the pairs in this study involved pairs of close relations.
Despite a lack of inbreeding, our population experiences high rates of hatching failure compared to a continent-wide survey of eastern bluebirds (Cooper et al. 2006). In addition to inbreeding effects, temperature and humidity also influence hatching failure (Cook et al. 2005), and we have observed that rates of egg hatching failure are positively related to relative humidity (Liu et al. Chapter 5). Thus, environmental factors might out-weigh any genetic factors influencing hatching failure.

The importance of avian genetic compatibility on fitness has received attention recently but results are equivocal (Mays et al. 2008). This phenomenon has been argued to be more common in more isolated and inbred populations. For example, isolated, inbred island populations of Seychelles warblers (*Acrocephalus sechellensis*) experience lower reproductive success (Richardson et al. 2004) and offspring of more-inbred song sparrow (*Melospiza melodia*) pairs are less likely to survive (Reid et al. 2005). Evidence for heterozygous advantages in healthy wild populations are less obvious (Hansson and Westerberg 2002). Two explanations for lack of correlation between heterozygosity and fitness in natural populations are the local effect and the general effect hypotheses. The local effect hypothesis argues that the apparent heterozygote advantage at the markers accurately reflects degree of heterozygosity of closely-linked fitness loci (Pemberton et al. 1991; Pemberton et al. 1988) while the general effect hypothesis argues that apparent heterozygote advantage at the markers is the result of effects of heterozygosity at genome-wide distributed fitness loci (Hansson and Westerberg 2002). For the microsatellite markers that we used in this study, the local effect hypothesis is the best explanation as we found that two of 10 loci (MOBL53 and SIALIA22) showed evidence of linkage disequilibrium. Whether the two microsatellite loci in our population that did not confirm to Hardy-Weinberg equilibrium are linked to fitness loci is worth further investigation.
We argue that the benefit of mating with a genetically more-compatible mate could be masked by environmental effects, especially in a territorial species in which variation in resources may trump genetic benefits (Griffith and Pryke 2006). The beneficial effects of genetic compatibility thus may often be difficult to detect in wild populations.

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Figure 4.1a),

b),
Figure 4.1

The relationship between female annual reproductive success and a) male UV-blue structural color: brightness (standardized for year effects), b) genetic compatibility index of pairs, and c) male heterozygosity index.
Figure 4.2 a),

The relationship between nestling mass at age 5 days and genetic compatibility of pairs in the a) early season ($P = 0.03$) and b) late season ($P = 0.6$).
Figure 4.3

The relationship between nestling mass at age 5 days and male plumage brightness in the a) early season ($P = 0.5$) and b) late season ($P = 0.001$).
Table 4.1 Characterization of 10 microsatellite loci for Sialia sialis (n=308 individuals genotyped). 

$N_a$: number of alleles; $H_O$: observed heterozygosity; $H_E$: the expected heterozygosity; HWE: test of deviation from Hardy-Weinberg equilibrium.

<table>
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<th>$N_a$</th>
<th>Individuals</th>
<th>$H_O$</th>
<th>$H_E$</th>
<th>HWE</th>
<th>$F$(Null)</th>
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*p<0.05
Table 4.2. Effects of male brightness (color) and genetic compatibility of pairs (GC) on nestling mass and wing length in the early and late season.

<table>
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<th>Trait</th>
<th>Age</th>
<th>Factors</th>
<th>Estimates</th>
<th>SE</th>
<th>df</th>
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<th>P</th>
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<td>1.244</td>
<td>4.99</td>
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<td>Color</td>
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<td>1.269</td>
<td>10.49</td>
<td>0.003**</td>
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Random effects: female ID
*p<0.05, **p<0.01
Chapter 5. Egg Viability in Relation to Laying Sequence and Incubation Behavior in Eastern Bluebirds
Abstract

Temperature is among the most critical factors affecting egg viability because embryos in the early stages of development are particularly sensitive to the thermal environment. A consequence of this thermo sensitivity is that the viability of unincubated eggs declines over time. The egg viability hypothesis proposes avian parents maximize the hatchability of eggs by initiating incubation before their clutch is complete. By using detailed temperature data from thermal data loggers placed in nests, we assessed the effect of laying order, seasonality, ambient temperature, and incubation behavior on hatchability of eggs in eastern bluebirds (*Sialia sialis*). We found that egg mortality was negatively related to position in the laying sequence, with earlier-laid eggs experiencing three times greater mortality rates than last-laid eggs. Contrary to our predictions, however, we found no evidence that hatching failure was related to nest temperature in the pre-incubation or early incubation stages. We did find, however, that temperature during the late incubation stage was positively related to hatching success. Additionally, we used a larger dataset to test whether hatching success was predicted by local temperature and humidity during the pre-incubation period, as well as seasonality, year, and clutch size. Of these variables, only ambient humidity predicted hatching failure. Our results suggest that temperature is not the major factor determining egg hatchability during the pre-incubation and early incubation period. The relationships between egg mortality, egg laying sequence, and ambient humidity suggests that high humidity during the pre-incubation period exposes early-laid eggs to environmental factors (e.g. microorganism infections) that increase hatching failure in our Alabama population of eastern bluebirds.
Introduction

Hatching failure is common across a wide range of bird species (Koenig 1982, Cooper et al. 2006). Failure of eggs to hatch could be caused by external factors such as unfavorable temperatures that could induce embryo mortality (Stoleson and Beissinger 1999), infection by microorganisms (Cook et al. 2005a) or internal factors such as genetic incompatibility (Hansson 2004), or insufficient sperm to fertilize eggs (Potti and Merino 1996). From geographical data, several studies have found that the probability of hatch failure varies with latitudinal and seasonal gradients (Cooper et al. 2005, reviewed in Koenig 1982). In eastern bluebirds (*Sialia sialis*), for example, hatching failure is greater in lower latitudes and later in the breeding season (Cooper et al. 2006). These latitudinal and seasonal patterns in hatching failure are thought to be caused by increasing temperature.

Temperature is considered one of the most critical factors affecting egg viability as embryos in the early stages of development are particularly sensitive to the thermal environment (Webb 1987). Most passerine birds commence incubation behavior on the day they lay the penultimate or ultimate egg (Deeming 2002). This late onset of incubation leaves earlier-laid eggs exposed to ambient temperature, and embryos may begin to develop if the ambient temperature reaches 24-26°C (the physiological zero temperature) or above (Webb 1987). During the incubation period, eggs need to be maintained at an optimal development temperature (36-39°C, White and Kinney 1974). The temperature range between 25°C to 36°C is considered as sub-optimal for embryo development. Exposure to these sub-optimal and unstable ambient temperatures can cause developmental problems, and increase the likelihood of egg death. The egg viability hypothesis proposes that avian parents maximize egg hatching success by initiating incubation before the clutch is completed (Hussell 1985, Webb 1987, Veiga 1992, Stoleson and
Beissinger 1999). An experimental study of a tropical passerine suggests that pre-incubation exposure to ambient conditions increases egg mortality (Stoleson and Beissinger 1999). Although temperature is thought to be the most critical factor affecting hatchability of un-incubated eggs, humidity, gaseous environment, and microbial infection have also been shown to be important, at least under some conditions (Wilson 1991, Deeming 1992, Fasenko et al. 1992, Meijerhof 1992, Cook et al. 2003, Cook 2005a). Many experimental tests by poultry researchers have investigated the relationship between temperature and hatching success in domestic chickens, but relatively little research has focused on the effects of incubation behavior on egg viability in wild birds (Deeming 2002).

The goal of this research is to determine the importance of nest temperature on hatching success using a population of eastern bluebirds breeding in Alabama, N.A.. To this end, we investigated three lines of evidence that may be related to hatching failure 1) within-clutch laying order, 2) seasonal changes in ambient temperature and humidity, 3) nest temperature throughout the incubation period.

According to the egg viability hypothesis, if early exposure to high ambient temperatures causes hatching failure, we can make the following predictions. First, within a clutch, we predict that earlier-laid eggs will experience higher hatching failure than later-laid eggs because they are more likely to experience suboptimal temperature in the pre-incubation period. Second, clutches exposed to higher ambient temperatures during the pre-incubation period will experience increased hatching failure. Third, clutches laid later in the season will experience greater hatching failure than earlier-laid clutches. To our knowledge, this is the first investigation of detailed measures of incubation temperature and egg viability in a wild-breeding population.
Methods

Eastern bluebirds are common, obligate cavity-nesting passerines found in open country throughout most of eastern North America. Eastern bluebirds exhibit a seasonal decline in clutch size (Gowaty and Plissner 1998) and, in the more southern latitudes, bluebirds lay smaller clutches (Dhondt et al. 2002) and experience lower hatching success (Cooper et al. 2006). We studied a population as part of a long-term research program in Auburn, AL. In this population, the clutch size ranges from 2-7 eggs, with a modal clutch size of 5. Bluebirds in this population commonly produce two successful broods during a breeding season that lasts from mid-March to early August (Siefferman and Hill 2005). This population nests in man-made nest boxes in pastures and grassy fields along forest edges. Nest boxes are on 1.5 meter poles and made of pine with ventilation following specifications of the North American Bluebird Society (http://www.nabluebirdsociety.org/eastwestbox.htm).

We monitored the breeding behavior of this population (230 nest boxes and approximately 150 nesting pairs per year) throughout the entire breeding seasons of 2005 and 2006. Each individual was marked with a unique combination of three color bands and a U.S. Fish and Wildlife service aluminum band (for detailed protocol of capture and measurement see (Siefferman and Hill 2003)). We visited active nest boxes every 3 days during the nest construction period. During the egg-laying period, we visited the nest daily and labeled each freshly-laid egg on the blunt side with a permanent marker (Sharpie®, USA).

We obtained climate data from the Quality Controlled Climatology Data of National Climate Data Center (NCDC, www.ncdc.noaa.gov) from the nearest climate station to our study site, the Auburn-Opelika Airport (station number: 03892), ~7 km from our study site. Using the controlled quality hourly data of relative humidity and air temperature (Dry ball temperature), we
averaged data from the first 4 days after the first egg date of each clutch to represent the climate that the clutch experienced during the egg-laying period.

In a subset of clutches (n = 45), we positioned temperature data loggers (Thermocron iButton®, Dallas Semiconductor, Dallas, TX, accuracy: ±0.5°C; Texas Instrument, USA) just beneath the egg surface, and well-embedded in the nesting material on the day that females laid the second egg of the clutch. We used monofilament fishing line to secure the data loggers in position throughout the incubation period. Temperature sensors were programmed to record surface temperature every 3 min and logged temperature readings throughout the egg laying and incubation period, thus minimizing disturbance to the nests. Nests were randomly selected across the breeding season each year. For the majority of breeding pairs, incubation temperature was measured one time, although the nests of a few pairs were measured twice.

Eastern bluebirds are obligate female intermittent incubators, therefore the egg temperature undergo cycles of cooling and heating during the incubation period (Gowaty and Plissner 1998, this study). We plotted temperature data (°C versus time) to determine female incubation activity before further analysis (Fig. 1). Temperature data from four nests exhibited signs that females had abandoned nests and were excluded from analyses. All remaining nests shared a common signature of female incubation activity. During the daytime, temperature readings raised abruptly reaching 32-34°C, suggesting that females were incubating eggs. These temperatures were generally sustained for 20-40 min and then the temperature dropped precipitously. After a period of time lasting between 10-20 min, the temperature rose again creating an U shape curve. We interpret this thermal signature as a resumption of incubation behavior, and these thermal curves during incubation are similar to those of other species that are intermittent incubators (Hainsworth and Voss 2002). We define this incubation- nonincubation-
incubation thermal signature as an ‘incubation bout’. During the night, nest temperature showed an unbroken flat line (32-34°C), suggesting that females constantly incubate eggs.

In general, eastern bluebirds lay one egg per day, so it takes 5 days to complete a five-egg clutch. Incubation begins on the penultimate day of egg laying followed by 14 days of incubation before chicks hatch (Gowaty and Plissner 1998). We organized thermal data into three stages: pre-incubation period, early-incubation period, and late-incubation period (Fig. 1). We sampled the pre-incubation stage data from the date that the 2nd egg was laid until the day that the 3rd egg laid was laid; a 24 hr period. We sampled early-incubation temperature data from the date that the 4th egg was laid through the next 7 days. We sampled late-incubation temperature data from the date at the end of the early-incubation stage through the next 7 days (Fig. 1). During the pre-incubation stage, we calculated five parameters to represent aspects of incubation temperature: 1) The maximum temperature (Tmax), 2) the average temperature (Tmean), and 3) the standard deviation of the temperature (Tsd). In the early- and late-incubation stages, we calculated two parameters from each stage: 1) the average temperature (Tmean), and 2) the standard deviation of the temperature (Tsd). Based on our thermal data, some females engaged in partial incubation behavior during the egg laying period. Therefore, we calculated 4) the number of incubation bouts during this 24 h pre-incubation period. Additionally, as ambient temperature above 25°C (physiological zero) may be harmful to eggs during the pre-incubation period (Decuypere and Michels 1992), we include 5) the percentage of time above 25°C. Because we have a smaller subset of data for number of incubation bouts and percentage of time above 25 °C, we do not include these variables in the main models. Instead, we use correlations to exam the relationships to hatching failure.
We used two different criteria to quantify hatching failure in each clutch. First, to examine the incidence of hatching failure in relation to incubation behavior and nest temperature, we divided all nests into two categories: nests in which all eggs hatched, and nests that contained at least one unhatched egg (Incidence of hatching failure = 0 or 1). Second, we calculated hatching failure for each nest as the percentage of eggs that remained unhatched (Percent hatching failure = number of unhatched eggs / total number of eggs in clutch). Finally, to determine whether hatching failure was related to egg-laying sequence, we calculated the percentage of unhatched eggs in each laying order.

Data analyses:

We tested normality of variables using Shapiro-Wilk tests. We first tested for effects of year, and when no year effects were significant, we combined the data from two different years. We calculated the mean air temperature and relative humidity during the egg laying period (4 days) of each clutch. It has been proposed that substantial decreases in clutch size with season might mask a true relationship between hatching failure and seasonality (Gowaty and Plissner 1998, Cooper et al. 2006). Indeed, we found that clutch sizes decreased as the season progressed (2005: r = -0.32, n = 88, P < 0.01; 2006: r = -0.43, n = 137, P < 0.001), thus we included clutch size as a covariate in the following analyses. Because the environmental factors were more likely to impact first-laid eggs (first-laid eggs experience the longest exposure without female attendance), we tested whether environmental factors influenced the hatching failure of first-laid eggs using backwards logistic regression. We ran separate models depending on whether we had collected 1) ambient climate data or 2) nest temperature data. The first group of predictor variables included year, first egg date, clutch size, average temperature during the egg laying period, and average relative
humidity during the egg laying period. The second group of predictor variables included first egg
date, clutch size and egg temperature data yielded from thermo sensors. We also investigated
whether these two sets of environmental factors influenced the hatching success of the entire
clutch using Spearman Rank correlations as the hatching success data were categorical. Finally,
because hatching success could be influenced by relationships between egg laying order, we used
an Pearson Chi-square test to examine the effect of laying order on hatching success. All statistical
analyses were performed using SPSS version 11.5 (SPSS Inc.).

Results

Clutch hatching failure

In 2005, we determined the egg laying sequence of 88 nests. Clutch size ranged from 2-6 eggs,
although the majority of nests had either 4 (41%) or 5 (44%) eggs. Fifty of 88 nests (57%)
contained at least one unhatched egg. In 2006, we determined the laying sequence of 137 nests,
and clutch sizes ranged from 3 to 6 eggs. The most common clutch sizes were clutches of 4 (39%)
and 5 (48%) eggs. Seventy two of the 137 nests (53%) contained at least one unhatched egg. In
both years, the incidence of hatching failure varied across clutches of different sizes (Table 1).

Hatching failure rate did not differ with year ($t = -1.37, df = 223, P = 0.17$) and we did not
detect a significant clutch size by year interaction ($F_{3,216} = 0.48; P = 0.7$). Therefore we either
combined both years of data or retained year as covariate in the following analyses.

Egg laying-order and failure rate

Hatching failure varied significantly with laying order ($X^2_{5,1004} = 39.4, P < 0.0001$; Fig. 2). The
first-laid eggs were the least likely to hatch; only 66.2% of $1^{st}$ eggs hatched (total $n = 225$), while
76.4% of 2\textsuperscript{nd} eggs (total n = 225), 81.2% of 3\textsuperscript{rd} eggs (total n = 223), 87.8% of 4\textsuperscript{th} eggs (total n = 205), 87.7% of 5\textsuperscript{th} eggs (total n = 114), and 100% of 6\textsuperscript{th} eggs hatched (total n = 10).

Ambient environment and hatching failure

In 2005, birds commenced egg laying between March 28\textsuperscript{th} until Jun 29\textsuperscript{th}; and, in 2006 from March 23\textsuperscript{rd} until July 17\textsuperscript{th}. Using the climate data obtained from the Auburn-Opelika weather station we found that, as the breeding season progressed, both average air temperature ($r = 0.73$; $n = 225$; $P < 0.001$), and relative humidity ($r = 0.36$; $n = 225$; $P < 0.001$) increased. Thus, the climate shifted from a relatively cool and dry environment to a relatively hot and humid environment.

Of all the predictor variables associated with local climate during the egg-laying period (year, first egg date, clutch size, mean ambient temperature, and mean relative humidity), only to relative humidity significantly predicted the fate of first-laid eggs (Final Model: $X^2_{1,225} = 6.09$, $P = 0.01$; Humidity: Wald = 5.85, $P = 0.02$). Our results remained the same when we analyzed the relationship environmental variables and percent hatch failure using Spearman Rank correlations; the only significant correlation was between relative humidity and hatching failure ($r_s = 0.14$, $n = 225$, $P = 0.03$). In both analyses, eggs were less likely to hatch in a higher humidity environment.

Nest temperature and hatching failure

In a sub-set of nests, we recorded the thermal environment during the incubation stage to investigate fine-scale nest temperate fluctuations in relation to hatching failure. Of all the predictor variables associated with nest temperature during the egg-laying period (year, first egg date, clutch size, pre-incubation maximum temperature (Tmax), pre-incubation mean temperature (Tmean), pre-incubation temperature standard deviation (Tsd), early-incubation Tmean and Tsd and
late-incubation Tmean and Tsd), only late-incubation temperature remained in the final model (Final Model: $X^2_{1,36} = 4.42, P = 0.04$; Late-incubation temperature: Wald = 3.4, $P = 0.06$).

First-laid eggs in nests with higher temperatures during the late-incubation period were more likely to hatch. Again, correlations suggest that only late-incubation temperature was significantly related to hatching failure; eggs were more likely to hatch in a higher temperature nest ($r_s = -0.33$, $n = 36, P = 0.04$). Additionally, because we had a smaller sample size of nests in which we recorded the number of incubation bouts and the percent time over 25°C during the pre-incubation stage, we isolated those two parameters from the main models and tested the relationship to the hatching failure separately. We found that neither the number of incubation bout ($r_s = 0.12, n = 20, P = 0.61$), nor the percent time over 25°C during the pre-incubation stage was related to hatching failure ($r_s = 0.20, n = 20, P = 0.41$).

Discussion

For the eggs of eastern bluebirds in Alabama, position in the laying sequence had strong effects on hatchability; hatching failure was highest in the first-laid egg (34%), and decreased as the laying order progressed (Fig. 2). Eggs exposed to ambient environments may suffer from high temperature and microbial infection facilitated by high humidity. Indeed, we found that the relative humidity measured from the nearest weather station was positively related to the likelihood that eggs would fail to hatch. We found no consistent relationship, however, between seasonality and hatching failure, nor did we find that pre-incubation nest temperatures predicted hatching failure.

Using detailed thermal data collected in the nests, we found no evidence that nests that experienced extreme temperatures prior to incubation (maximum, average, and duration above
25°C) experienced lowered hatching success. Across the entire incubation period, temperature late in the incubation stage was the only significant predictor of hatching success. Eggs in nests that experienced high temperatures were more likely to hatch. This higher temperature might be a consequence of heat generated by the developing eggs (Deeming and Ferguson 1992), rather than female incubation effort, as egg temperatures were higher during the late incubation period compared to the earlier stages. If thermal environment prior to incubation is a crucial determinant of hatching success of our population, we ought to have detected an increase in hatching failure as the season progressed. Hatching success, however, did not decrease with increasing season although ambient temperatures did increase throughout the summer. The egg viability hypothesis suggests that high ambient temperature and humidity are both potential hazardous factors on unincubated eggs (Stoleson and Beissinger 1999, Cook et al. 2005a). In our population, high humidity was a better predictor of hatching failure than ambient temperature.

In an experimental study of a tropical passerine, Stoleson and Beissinger (1999) found that hatching failure increased with increasing time that eggs were exposed to the ambient environment; egg hatching success decreased to ~70% after three days of exposure in the ambient environment. This trend is similar to our observations; the hatching success of first-laid eggs decreased to ~70% (3 days exposure before incubation started) compared to ~90% hatching success of the fourth-laid eggs (0 days exposure). The few other studies that have investigated relationships between the lengths of time that eggs were exposed and hatching success have found that prolonged exposure dramatically reduces hatching success (Arnold et al. 1987, Veiga 1992, Cook et al. 2005a). In our population of eastern bluebirds, females begin full-time incubation when the 4th egg is laid, thus the common exposure time for the earliest-laid egg is 2-3 days. This pre-incubation exposure to ambient conditions is relatively short compared to other species
(reviewed in Stoleson and Beissinger 1999) and may partially explain the lack of a statistical relationship between temperature and hatching success in eastern bluebirds.

The breeding range of eastern bluebirds is distributed widely across eastern North America. In a larger-scale study, Cooper et al. (2006) found the hatching failure increased in the more southern populations. Moreover, rates of hatching failure increased slightly as the breeding season progressed. These geographical and seasonal trends suggest that high ambient temperatures are responsible for hatching failure. Although our population experienced an extremely high hatching failure (53% of our clutches contained one or more unhatched eggs compared to the national average of 23% (Cooper et al. 2006)), our detailed temperature data indicate that pre-incubation ambient temperature is not responsible for the high incidence of hatching failure in this population. We also failed to detect a clear trend between seasonality and hatching failure. These contradictory results bring up two questions: 1) Why do we see differences between broad and local scale research efforts in the same species? 2) If not temperature, what is responsible for these latitudinal changes in egg viability?

Ambient relative humidity was the best explanation for hatching failure in our population. Clutches exposed to high relative humidity were more likely to experience hatching failure. Microorganisms might be responsible for the increased hatching failure of earlier-laid eggs. Bacteria and fungi were likely the cause of egg death in our population of eastern bluebirds in 2003 (M. D. Shawkey, unpub. data). Indeed, several studies have found that rapid growth of bacteria on eggshells might increase egg mortality in other species (Cook et al. 2003, 2005a). Moreover, experimental evidence suggests that microorganisms on eggshells are likely more detrimental in humid environments compared to arid environments. Cook et al. (2005a) conducted a manipulative experiment in which egg surface was either cleaned or remained naturally uncleaned
and these pre-incubated eggs were exposed an altitudinal gradient (humid and cool versus dry and hot environments) for 5 days. Although both environment and cleanliness of eggs had important impacts on hatching success, egg viability was lowest in uncleaned eggs that experienced the moist and cool environments (Cook et al. 2005a). It is likely that humidity strongly influences growth of bacterial on eggshells and thus increases the chance of egg infections. Moreover, species occurring in dry Mediterranean climate zones generally experience low hatching failure (Stoleson and Beissinger 1999). Perhaps the geographic variation in hatching success in eastern bluebirds, wherein hatching failure was highest in the lowest latitudes (Cooper et al. 2006), is driven more by increasing ambient humidity than by increasing ambient temperature. Interestingly, Cooper et al. (2006) report that the incidence hatching failure of eastern bluebirds dramatically shifts around 34° N latitude, rather than along a more gradual transition toward southern latitudes. Our study site (32° N) has much higher failure rates compared to other populations (Cooper et al. 2006); the relatively high humidity of our study site may account for this high failure rate.

Avian eggs appear to have a network of defenses against microbial infection; females may reduce the likelihood of hatch failure by commencing incubation earlier. Incubation of eggs reduces the excess moisture on the egg surface and higher temperatures activate anti-bacterial enzymes in the albumen thus reducing the exposure of eggs to trans-shell microbes (Cook et al. 2005b). Thus, incubation behavior may maintain the viability of eggs by activating antibacterial proteins (Tranter and Board 1984). In tree swallows, ambient temperatures are an important determinant of whether females initiate earlier incubation behavior (Ardia et al. 2006). This suggests that female incubation behavior may be a plastic response (Hebert 2002) to higher ambient temperature rather than fixed behaviors found in different geographic ranges. We found several females in our population that commenced partial incubation during egg laying period.
However, we did not find that this behavior was associated with ambient humidity. Whether this early incubation behavior is related to microclimate is worthy of further research.

Our results yielded mixed support for the viability hypothesis. Consistent with the hypothesis, eggs laid earlier in the clutch sequence experience higher mortality. We found no evidence, however, that high temperature was responsible for this within-clutch decline in hatching success. If exposure to the high temperatures caused egg mortality of earlier-laid eggs, we should have detected an increase in hatching failure with increasing pre-incubation nest temperatures. Surprisingly, neither higher pre-incubation temperatures nor seasonality predicted hatching failure. The most parsimonious explanation is that another factor (perhaps humidity) that eggs are exposed to during pre-incubation stages increases mortality rates of earlier-laid eggs. Future research should experimentally test the influence of humidity and cleanliness on eggshell microbes as well as the influence of shell microbes on hatching success in eastern bluebirds.

Acknowledgements

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References


Table 5.1 Incidence of hatching failure in relation to clutch size in eastern bluebirds in Alabama in 2005 and 2006.

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Figure 5.1

The typical temperature fluctuations of an eastern bluebird nest during the entire incubation period.

The pre-incubation period (egg laying) last 4 days, early- incubation period last 7 days, and the late- incubation period last 7 days.
Figure 5.2

Relationship between hatching failure and egg-laying sequence (white bars represent 2005, black bars represent 2006) in eastern bluebirds. The first-laid egg is represented by 1st.
Figure 5.3

Relationship between hatching failure and relative humidity in eastern bluebirds (2005 and 2006 data are combined)
Summary

In the past three decades, numerous empirical studies have demonstrated evidence of female mate choice for highly-ornamented male birds. It is argued that if ornamentation is an honest and reliable signal of male resource-holding ability or male genetic quality, females could either gain direct reproductive benefits or indirect reproductive benefits from mating with the most highly-ornamented males in the population. In particular, the good genes hypothesis emphasizes that genes for increased fitness could be identified by assessing male ornamentation, and that females that select highly-ornamented males would produce higher-quality offspring.

Studies of female mate choice in birds range from weak correlative field studies and uncontrolled aviary observations to carefully controlled experimental tests in both the field and the laboratory. Laboratory experiments can be useful because they allow researchers to manipulate coloration and disassociate color traits from other correlated traits. In my dissertation, I used a combination of aviary-based experiments, field experiments and correlational research to fully explore the importance of mate choice and environmental heterogeneity on reproductive success in eastern bluebirds. The first half of my dissertation (chapters 1, 2, and 3), I used aviary and field manipulations to tests the hypotheses that female mating preferences are based on male
plumage ornamentation and genetic characteristics. In the second half of dissertation (chapters 4 and 5), I examined whether male characteristics and environmental factors influence reproductive success.

In the first chapter, I examined female mating preferences in eastern bluebirds by utilized aviary-based mate-choice experiments. I allowed females to choose between males that had been artificially brightened and dulled to separate natural co-variation between male coloration and male quality. I followed this experiment with a similar design but I allowed females to choose between males with naturally brighter and duller blue structural coloration. I found no evidence that female eastern bluebird chose mates based on their structural coloration.

In the second chapter, I used a field-based design to again test female mate preferences for male ornamentation. To do this, I experimentally widowed males by capturing females and holding them in captivity. To control for the influence of territory quality on female choice, I widowed dyads of males with adjacent territories. In the field, new recruited and unmated females were able to choose between available males. The more-ornamented and larger males did not attract females more quickly compared to the less-ornamented and smaller males, thus I found no evidence that females preferentially settled with the more-ornamented males.

In third chapter, I used the same experimental design and tested the importance of male heterozygosity and the genetic compatibility of the pair on female preferences in the wild. I found
that females preferred to pair with the most genetically compatible mates. Additionally, my data demonstrate that females also tended to pair with the more heterozygous mates. Thus chapters 1, 2 and 3 suggest that female eastern bluebirds choose mates based on genetic characteristics and not ornamentation.

In the fourth and fifth chapters, I focused on reproductive success of wild breeding eastern bluebirds. In the fourth chapter, I used wild breeding birds to test the hypotheses that male ornamentation or genetic compatibility of mates influences reproductive success. I found that females mated to brighter males fledged more offspring annually and that both male coloration and genetic compatibility of pairs predicted offspring quality. The relative importance of genetic compatibility versus mate ornamentation on nestling quality, however, varied with season. For first clutches, early in the breeding season, genetically more-compatible pairs produced heavier nestlings. On the other hand, for second clutches, later in the season, brighter males produced offspring that were heavier and had longer wings at fledging.

This Alabama population suffers high egg hatching failure. In the fifth chapter, I explored environmental factors that influence hatching failure. I found that position in the laying sequence had strong effects on hatchability; hatching failure was highest in the first-laid egg (34%), and decreased as the laying order progressed. Eggs exposed to ambient environments may suffer from high temperature and microbial infection facilitated by high humidity. Indeed, I found that the
relative humidity measured from the nearest weather station was positively related to the
likelihood that eggs would fail to hatch. I found no consistent relationship, however, between
seasonality and hatching failure, nor did I find that pre-incubation nest temperatures predicted
hatching failure. These data suggest that reproductive success of eastern bluebirds breeding in
lower latitudes may be more negatively influenced by high humidity than by high ambient
temperatures.

Although I found no evidence that females chose mates based on ornamentation, females
mated with the most-ornamented males often experienced higher reproductive success. It is likely
that ornamentation does not function to attract females but may function as a signal to other males
of resource-holding ability. Females mated to brighter males would be expected to experienced
higher reproductive success if more-ornamented males defended higher quality territories. Female
bluebirds did appear to choose mates based on the underlying genotype and those females mated
with the most genetically compatibly mates achieved higher reproductive success. Overall, my
dissertation suggests that environmental variability, mate ornamentation, and genetic
compatibility of mates all influence reproductive success in eastern bluebirds.