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ENVIRONMENTAL VARIATION AND SEXUAL SELECTION IN THE MOUNTAIN BLUEBIRD (SIALIA CURRUCOIDES)

Sara A. Berk
University of Montana, Missoula

ENVIRONMENTAL VARIATION AND SEXUAL SELECTION IN THE MOUNTAIN BLUEBIRD (*SIALIA CURRUCOIDES)*

By

Sara A. Berk

BA Ornithology, Ecology, and Physiology, Hampshire College, Amherst, MA 01002

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Approved by:

Advisor: Creagh Breuner

Committee:

Doug Emlen

Tom Martin

Nathan Morehouse

Bret Tobalske

Abstract

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Environmental variation and sexual selection in the mountain bluebird (*Sialia currucoides)*

Chairperson: Dr. Creagh Breuner

Sexual selection acts on traits that increase mating success, either through mating preferences or intrasexual competition for access to mates. For traits to be honest, we expect sexually selected traits to reflect individual condition at the time of trait development. Furthermore, when sexual selection operates through mating preferences, we also expect traits to indicate benefits (direct or indirect) that females receive for exercising their preference. If sexual selection acts through differential success in intrasexual contests over mates, we expect traits to indicate resource holding potential, or fighting ability. These links between individual condition, trait quality, and performance maintain honesty, because high condition individuals have high quality traits, and conspecifics can therefore use information from sexually selected traits when entering contests or choosing between prospective mates.

Based on the above logic, we expect sexually selected traits to be consistently and positively related to performance. However, individuals may differ in their sensitivity to environmental variation such that sexually selected traits are not always honest indicators of individual condition, benefits to females or offspring, or competitive ability. Environmental variation could affect trait honesty if individuals vary in their ability to respond to environmental variation. For example, trait honesty may disappear in poor environments, if individuals with highly developed sexually selected traits only perform well in high quality environments. Alternatively, individuals with more elaborate traits may be more adept at responding to environmental challenge, and trait honesty could increase when environmental conditions are poor.

For my dissertation I examined variation in trait development and honesty under varying conditions in the mountain bluebird, *Sialia currucoides.* Mountain bluebirds display

sexually dimorphic UV-blue coloration, and males with more intense coloration sire more offspring at their own nest and at other nests through extra-pair fertilizations. However, it is unclear what benefits and costs receivers experience when using this trait to asses mates or rivals, and what processes regulate the development of this sexually selected trait. Therefore, in chapter one I explored the function of this signal during agonistic contests for territories. I performed simulated territorial intrusions to understand whether male aggressive behavior was related to his coloration. I also measured food availability to determine if males with more intense coloration obtained higher quality territories. Overall, my results provide evidence for the function of this signal during agonistic contests. Furthermore, while a single aggressive behavior (number of attacks) was repeatable across the egg laying period, my integrated metric of aggression, which accounted for many aggressive behaviors and was related to male coloration, was not. Lastly, I found that males with more saturated coloration obtained territories with greater insect abundance.

In chapter two, I performed an experiment to understand the mechanisms of condition dependence of blue coloration. Individuals may vary in their sensitivity to environmental variation during trait development; such high condition individuals preserve trait quality during environmental challenge while poor condition individuals do not. Martin et al. (2011) suggest that endocrine systems are an important mediator of phenotypic variation because hormones both respond to environmental conditions and regulate internal response and resulting phenotype. The hormone corticosterone (CORT) is released by the adrenal glands in response to challenge to divert resources towards self-preservation. A rapid, transient increase in CORT can help individuals to survive challenging situations. However, prolonged or frequent CORT secretion can cause damage to other physiological systems and potentially decrease fitness. For example, CORT-implanted white crowned sparrows abandon their high-elevation breeding territories and retreat to low elevations during storms. This increases survival but decreases reproductive success. Also, CORT-implanted male song sparrows increase fat stores, but are less likely to respond aggressively to a simulated territorial intrusion. These links between environmental conditions and potential fitness consequences make CORT an ideal regulator of sexually selected traits.

To this end, I brought 14 hatch year mountain bluebirds into captivity to study how individual variation in CORT physiology during resource limitation predicted coloration. I wanted to understand whether CORT predicted blue coloration when resources were abundant, limiting, or both. I found that when birds were food limited, variation in CORT levels increased. Furthermore, CORT and coloration were negatively associated in my food limitation treatment, as predicted if poor condition males mount larger stress responses, but this association disappeared when birds were given ad lib food. I also measured feather structure variables to determine how coloration was related to feather quality and feather performance (measured as resistance to airflow) across my resource availability treatments. I found that the component of feather structure that was related to coloration (barbule density) was sensitive to CORT only when resources were limiting. Conversely, the feather structure variable that determined feather performance (interbarb distance) was not sensitive to CORT in either treatment and did not predict feather coloration. These results indicate that feather coloration in bluebirds is only sensitive to variation in physiology when resources were limiting, and that this was mirrored by concurrent changes in condition-sensitive feather structure. However, feather performance was not sensitive to individual variation in physiology or variation in environmental conditions.

While chapters one and two demonstrated that blue coloration is condition dependent and related to aggressive behavior, male bluebirds still provide a significant amount of parental care. This means that females may be using coloration to select mates and obtain benefits for themselves and their offspring. In chapter three, I examined the effect of environmental variation on the relationship between bluebird coloration and direct benefits to females, expressed as offspring quality (nestling mass). Three years of data on free-living bluebirds suggests that the relationship between male coloration and nestling mass varied across years and between broods. In some contexts, more elaborate males had heavier nestlings, while in other contexts they raised the lightest nestlings. I found that this variation was not driven by resource abundance, but instead appeared due to changes in optimal reproductive effort. When average nestling mass at my study site was higher, bluer males raised heavier nestlings than they did when average nestling mass was lower. This variation in nestling mass occurred independently of resource availability.

Overall, these results demonstrate that the honesty of blue coloration varies across environmental contexts. While coloration is positively related to aggressive behavior, males vary in their sensitivity to resource limitation, and blue coloration does not consistently predict direct benefits in the wild. My data indicate that the process of sexual selection is context-dependent, and sexually selected traits vary in the extent to which they predict individual performance and quality.

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Chapter 1: Coloration signals aggressive behavior and territory quality in the mountain bluebird , *Sialia currucoides*

Abstract

Sexual selection is an evolutionary force that can result in highly elaborate traits. These traits function to increase mating success through intrasexual or intersexual competition. We studied whether blue coloration in the mountain bluebird (*Sialia currucoides)* is relevant during intrasexual contests over nest sites. Sexually dimorphic blue coloration in this species has been linked to mating success, but we know little about the function of this signal during aggressive interactions between males. Coloration may signal status and resource holding potential, but it is unclear whether aggressive behavior is based on individual status, the status of a competitor, or mutual assessment of both. We performed simulated territorial intrusions to understand whether male aggressive behavior was linked to his own status, the status of the simulated intruder, or both. We also measured food availability to determine if males with more intense coloration also obtained higher quality territories, as would be expected if plumage brightness honestly signals male resource holding potential (RHP). We found that male aggressive behavior was positively related to his own coloration, but not the coloration of his simulated opponent. However, while a single aggressive behavior was repeatable, our integrated metric of aggression was not. Lastly, we found that males with more saturated coloration obtained territories with greater insect abundance. Overall, our results provide evidence for the function of this signal during agonistic contests, and for the honesty of brightness as an indicator of male resource holding potential.
Key words: sexual selection, behavior, territory quality, aggression

Introduction

Sexual selection is a complex evolutionary process resulting in elaborate traits that can increase mating success (Andersson 1994). First, sexually selected traits are utilized during intrasexual contests for mates. This type of sexual selection results in weapons that are directly used in fighting or signals of status that competitors use to evaluate each other. Second, sexually selected traits can be used intersexually during mate choice (Andersson 1994, Darwin 1859, 1871). Conspecifics use signal traits to select mates that will provide benefits either directly,

through resources delivered during the current generation, or indirectly to future generations through "good genes" effects (Andersson 1986, Kirkpatrick 1982, Lande 1981, Møller and Jennions 2001, Moller and Alatalo 1999, Neff and Pitcher 2005).

During intrasexual competition, rivals can use status signals to evaluate the fighting ability of their opponent before escalating contests and avoid fights they are unlikely to win (Maynard Smith 1982, West-Eberhard 1979). These ornamental signals of status are distinct from weapons because they are used during rival assessment but are not directly utilized during fights (McCullough et al. 2016). Therefore, investigating whether status signals reflect fighting ability is a crucial component of demonstrating the information content of signaling traits (Searcy and Nowicki 2005). A great deal of research in birds and other taxa has indicated that ornamental traits are related to resource holding potential (RHP) and fighting ability (Hughes 1996, Otter et al. 1997, Parker 1974, Pryke and Andersson 2003, Pryke and Griffith 2009, Rohwer and Rohwer 1978). Dark-eyed juncos with experimentally decreased plumage quality are less likely to win fights with other males (Grasso et al. 1996). Furthermore, *P. dominulus* paper wasps preferentially select rivals with facial patterns associated with lower quality (body size) when choosing individuals to challenge for contests over food (Tibbetts and Lindsay 2008). These results demonstrate that signal traits can be correlated with fighting ability and that receivers can distinguish between individuals when choosing to escalate contests.

However, we have less evidence for relationships between fighting ability and contest outcome in natural populations. For example, signal quality may be related to territory quality or mate access for reasons other than RHP if higher quality males arrive to breeding sites earlier and avoid competition. Linking performance during fights to resource-winnings will allow us to better understand trait honesty and the maintenance of positive selection on sexually selected traits. We explored whether the sexually dimorphic UV-blue coloration in mountain bluebirds (*Sialia currucoides)* is informative between males during competition for nest sites. Previous studies have found that male mountain bluebirds with more elaborate coloration sire more offspring (Balenger et al. 2008). However, it is unclear what behaviors or mechanisms (intrasexual vs. intersexual) lead to this increase in reproductive success. Mountain bluebirds must nest in a cavity, but cannot excavate their own, leading to intense competition for nest sites during the early spring. Male eastern bluebirds with more elaborate coloration obtain nest boxes earlier when territory availability is experimentally limited, indicating that this signal may be

related to competitive ability (Siefferman and Hill 2005). However, to date, no study has observed whether resource holding potential or aggressive behavior of bluebirds covaries with male coloration.

To this end, we performed simulated territorial intrusions to determine if male aggressive behavior was predicted by male coloration, and whether male behavior was repeatable across multiple observations. We also assessed whether coloration was related to contest outcome by measuring male territory quality (insect abundance). Our results shed light on the function of coloration in this system, and the mechanisms through which competitors determine the degree of contest escalation.

Methods

Field Site

We studied mountain bluebirds outside of Ronan, MT on the Flathead Indian Reservation (47.478370, -114.377034) from March 20th to April 20th, 2016 and March 28th to May 3rd, 2017. The study site consists of 45 nest boxes spread across seven miles of fence line on a dirt road through sagebrush habitat. We scored nest development on a scale of 1-4 and checked highly developed nests (score 3 or 4) every other day until first egg.

Simulated Territorial Intrusions

In 2017, we conducted simulated territorial intrusions (STI) on resident males (hereafter called 'focal males'; n=44 trials at 25 nests) at the discovery of first or second egg. For a subset of nests discovered on the first egg (n=14), we repeated STIs after the appearance of the third and fifth egg using a separate specimens to measure both the repeatability of the response of the focal male as well as how his response changed with specimen color (see specimen source description below (n=5 nests repeated twice, n=9 nests repeated three times). We did not visit nests on the days between repeated intruder trials. During the first trial, we used a random number generator to select one of our eight available specimens. For repeated trials, we divided specimens into "least chromatic," "most chromatic," and "medium chromatic" groups using spectrophotometry data (see below). We ensured that males receiving three trials encountered the full range of available specimen coloration by restricting our random selection to novel groups during repeated trials. For example, if a male was shown one of the "most chromatic" specimens during

his first trial, we restricted our random number generation so that he was not presented with another highly chromatic specimen during his second or third trial.

Specimen Sources: For specimens, we used eight taxidermy birds prepared from our captive population. These birds had been raised in captivity during summer 2014 and summer 2016. If a specimen was damaged during trials, we either repaired it using super glue or discontinued use.

Trials: We conducted ten-minute STI trials at each nest. We attached specimens to a 60-inch wooden dowel affixed to a camera tripod. We also attached two other wooden dowels to the tripod to allow the focal bird places to perch during the trial. We painted the tripod and dowels green to decrease conspicuousness. We set the tripod and specimen 3m from the front of the nest box next to the fence line. The observer (always SB) covered the specimen with a cloth attached to a fishing line and retreated to a location at least 20m from the nest box. After a five-minute waiting period, the observer pulled the cloth from the specimen and began a three-minute playback period. For playback, we used an iPhone 6 plus at max volume concealed directly underneath the tripod. To avoid disturbing the birds to begin playback, we used a five minute recorded silence track such that the playback would automatically begin after five minutes. We only used one mountain bluebird call for all trials to limit specimen signal variation to visual cues. We recorded behavior of the focal male including latency to respond to the specimen, hovers directed toward the specimen, aggressive flights toward the specimen (males often display using undulating flight with no physical contact), and number of attacks to the specimen (these involved physically contacting the specimen with feet or beak). We also measured the number of times males perched at the entrance to the nest box, likely a defensive behavior (SB pers. obs.); males often appeared to be blocking the nest box entrance with their bodies while oriented towards the specimen. Males did not enter the nest box during any of our trials, though females often did. Finally, at the start of each minute we noted the position of the male and female as perched on the tripod with the specimen, within five meters of the specimen, within ten meters of the specimen, present within view but greater than ten meters from the specimen, or absent from the observer's field of view. We used flagging tape affixed to the fence around the nest box to allow for easy quantification of the position of the male and female relative to the specimen.

Territory Quality

We assessed territory quality during 2016 and 2017 using pitfall traps placed within 20m in each direction of the nest box along the fenceline. Mountain bluebirds are primarily pounce foragers and eat insects off the ground, rather than flying insects (Herlugson 1982). In 2016, we placed traps within 2 days of the appearance of the first egg at each nest (beginning April 2nd). Birds arrived to the study site later in 2017 (first egg = March 29th 2016, April 6th in 2017), so we began placing pitfall traps on April 4th, 2017 as nests reached later stages of development prior to egg lay. We dug holes 10cm deep and placed 10cm plastic cups into the hole before packing dirt back around the trap. We filled cups with approximately 4cm of 50/50 propylene glycol and water. Every 7-8 days we collected insects from traps and replaced the trapping liquid. We froze samples in plastic bags until analysis. After thawing, rinsing and sorting, we dried insects for five days at 60 degrees Celsius and weighed insects (by family group) to the nearest milligram. To obtain insect biomass, we sorted insects to include only those which are frequently eaten by mountain bluebirds; *Orthoptera, Coleoptera,* and *Lepidoptera*. We categorized *Coleoptera* and *Lepidoptera* into adults and larvae and weighed them separately. We obtained relative insect abundance of the breeding season by z-scoring samples collected on the same day so that they were centered at 0 with a standard deviation of 1. This allowed us to assess whether more colorful males had relatively higher quality territories for a given day of the season.

Color Measurement

We measured the color of rump feathers collected during capture using a USB4000 spectrophotometer with a pulsed xenon light source (Ocean Optics, Dunedin, USA). We took five reflectance measurements each consisting of ten averaged curves. We stacked seven feathers on top of each other and taped them to non-reflective black paper (Canson) for measurement. We positioned the probe at 90 degrees using a probe holder and standardized the distance between the probe and the specimen at 5mm. We standardized measurements between individuals using a white standard (Labsphere, NH), and turned off the light source and covered the probe to create a dark standard. To minimize variation we measured coloration of all males in a single day. Past measurements of repeatability of color measurements from the same observer (SB) in our lab indicate low coefficients of variation even when feather samples from a single individual are

measured several years later (CV Hue=6%, CV Brightness=9%, CV spectral saturation=4%, CV UV Chroma=4%).

To extract color variables, we averaged the resultant reflectance measurements (between 300 and 700nm) and smoothed spikes from curves using the program CLR 5 (v. 1.05, Montgomerie 2008). From these averaged curves we used R (R Core Team 2017) to extract the hue (wavelength of peak reflectance), blue chroma (proportion of the reflectance concentrated from 400-512 nm), UV-chroma (proportion of the reflectance concentrated from 300-400nm), and brightness (sum of the total reflectance). For wild birds, we also measured the spectral saturation (proportion of the reflectance concentrated within 100nm of the hue).

Analysis

We performed all statistical analyses using R (R Core Team 2017). We analyzed STI behaviors into a principal components analysis (PCA). We combined presence data into total time spent within 10m of the nestbox vs. greater than 10m or absent. However, we only included the time that males spent within 10m of the nest box in our PCA scores, to avoid the use of two binary variables. We scaled all behavior variables in our PCA to a mean of zero and standard deviation of one. All aggressive behaviors loaded positively onto the first principal component (PC1), which explained 48% of the variance in aggressive behavior (Table 1). In sum, males with higher aggression scores spent more time within 10m of their nestbox and performed more aggressive behaviors towards the specimen during our 10-minute observation period. We used only the first observation from each nest to evaluate color as a predictive factor for aggressive behavior (n=20). We used linear regression models and assessed model fit and assumptions using appropriate diagnostic plots and R^2. For analyses of the effect of specimen coloration on focal male behavior, we included all observations and fit a mixed effects model (R package nlme) with a random effect of male ID (n =14 separate focal males).

We used the package "rptR" to analyze repeatability of aggressive behaviors of individual males as well as responses to specimens (Nakagawa and Schielzeth 2010)**.** We bootstrapped repeatability estimates with 1000 iterations and specified a gaussian distribution for all repeatability analyses. We did not scale behavior variables when analyzing the effects of specimen coloration or repeatability of focal male behavior. To evaluate whether coloration was involved in obtaining higher quality territories, we used a mixed effects model including a

random effect of male ID to assess the correlation between male coloration and territory quality at the beginning of the season using the first pitfall trap we collected on his territory in each year (n=20 nests in 2016, n = 21 nests in 2017).

Results

Coloration and Aggression

In all of our analyses, male blue chroma and spectral saturation were the only significant predictor of focal male behavior. Male blue chroma was positively correlated with his response to the simulated territorial intrusion (Figure 2, β=0.40 $F_{1,14}$=8,p=0.01,R^2=0.36). Spectral saturation and UV Chroma were also positively related to male aggression (Saturation: β=0.41,$F_{1,14}$=11.89, p<0.01,R^2=0.42, UV Chroma: β=0.27, $F_{1,14}$=5.38, p=0.04,R^2=0.28). Hue and brightness were unrelated to male behavior (Hue: β=-0.04, $F_{1,14}$=2.92,p=11,R^2=0.17, Brightness: β=-0.01, $F_{1,14}$=0.144, p=0.71,R^2=0.01) These relationships were not due to seasonal effects, as there was no relationship between male coloration and nest initiation date across our study site (LMM: β_{color}= 0.11, $F_{1,67}$=0.03, p=0.85).

Territory Quality

Male coloration predicted the quality of his territory at the beginning of the season in both 2016 and 2017 (Figure 4**,** LMM: $F_{1,48}$=8.91, β=0.27, t=4.45, p=0.01). While the association between male coloration and insect abundance became less strong during the nestling phase (LMM: $F_{1,48}$=4.74, β=0.10, t=2.18, p=0.03), relative insect abundance was moderately repeatable across individual nests (R_{2016}=0.44, bootstrap 95% CI: (0.27,0.61), R_{2017}=0.39, bootstrap 99% CI: (0.24,0.53)). Territory quality was also not directly related to male aggression (β=0.08, $F_{1,12}$=0.13, p=0.73,R^2=0.009), or nest initiation date (LMM: β=0.01, $F_{1,43}$=1.90, p=0.17).

Repeatability of Aggressive Behavior

Repeatability analysis demonstrated that our aggression score (PC1) was not repeatable across the laying period (R=0.182, bootstrap 95% CI: (0,0.53), n=37 trials at 14 nests). However, we found that the number of times a focal male attacked the specimen was significantly repeatable (R=0.78, bootstrap 95% CI: (0.49,0.91)). Other behaviors were not repeatable among trials of the same focal male (aggressive flights: R=0.05, bootstrap 95% CI: (0,0.41), hovers: R=0.07,

bootstrap 95% CI: (0,0.45), perches: R=0.21, bootstrap 95% CI: (0,0.55), time within 10m of specimen: R=0.22 bootstrap 95% CI: (0,0.54)). Furthermore, focal male response was not repeatable based on the specimen used (first trials only, PC1: R=0, bootstrap 95% CI: (0,0.44), attacks R=0, bootstrap 95% CI: (0,0.40), hovers: R=0, bootstrap 95% CI: (0,0), aggressive flights: R=0, bootstrap 95% CI: (0,0.28), perches: R=0, bootstrap 95% CI: (0,0), time within 10m of specimen: R=0, bootstrap 95% CI: (0,0).

Discussion

Sexual selection operates through intrasexual competition and intersexual mating preferences **(**Andersson 1994**)**. Understanding how traits function during contests is crucial to comprehending the mechanisms of intrasexual selection (Hunt et al. 2009, Qvarnström and Forsgren 1998). We found that males with more chromatic coloration obtained higher quality territories. Across many bird species, males with more intense coloration have higher quality territories; such as nest sites that are more protected from predation, or that have increased food abundance (Hasegawa et al. 2014, Hill 1988, Keyser and Hill 2000, Wolfenbarger 1999). Plumage coloration can also signal territory quality during the nonbreeding season; male redstarts overwintering in high quality habitat have brighter tail feathers than males that overwinter in low quality second-growth habitat (Reudink *et al.*, 2009). Our results support further links between coloration, RHP, and territory quality as the result of success during intrasexual contests.

Our study design was such that resident males likely perceived their territory to be high value; we performed our observations when males had already established and been defending territories for some time. Our results demonstrate that blue coloration is informative during agonistic interactions. A more elaborate male is more likely to defend his territory against intruders, and this should reduce his and his mate's likelihood of losing their territory after establishment. From the perspective of rivals, this may reduce their propensity to enter into aggressive interactions with highly colorful males that own territories.

Across species, males with more elaborate plumage coloration often have higher reproductive success (Balenger et al. 2009, Brommer et al. 2005, Doucet et al. 2005, Hill 1988, Keyser and Hill 2000, McGraw et al. 2001, Safran and McGraw 2004, Siefferman and Hill 2003, Wiehn 1997). Given the widespread associations between plumage color and territory quality, it

is unclear if the relationship between coloration and reproductive success is due to its function as an agonistic signal or a target of female preference, or both. Previous work on eastern bluebirds (*Sialia sialias*) found that females did not display preferences for male coloration in laboratory preference tests (Liu et al. 2007). Also, more colorful males did not attract new mates faster than their less colorful neighbors after mate removal in the field (Liu et al. 2009). These results, when taken together with the data we present in this paper, suggest that bluebird coloration is primarily an agonistic signal representing possible aggressive response to conspecific challenge.

However, male mountain bluebird coloration positively predicts his total reproductive success (Balenger et al. 2008), and so this signal, or some correlate of it, may be used in female mate choice. Females may not always base their mating choices directly on male traits, but could choose aspects of the male's extended phenotype that result from winning competitive interactions with other males (Qvarnström and Forsgren 1998). High quality territories are one component of a male's extended phenotype that can provide increased resources for females. For example, female fish often choose spawning sites that are defended by high quality males. If males disappear or are experimentally removed, females will often remain at their spawning site rather than choosing a new mate (Jones 1981, Warner 1987). Traits that are used during intrasexual contests can therefore increase mating success even in the absence of strong female preference for that trait if females gain resources from mating with highly competitive males (Berglund et al. 1996, Qvarnström and Forsgren 1998). Females may receive benefits from exercising choices for more elaborately colored males, but further study is needed on the specific targets of female choice in bluebirds and other territorial species that utilize status signals.

Selection is unable to act on traits that are not a repeatable aspect of individual phenotype (Boake 1989). Interestingly, we found that some, but not all, components of a male's behavioral response to a simulated intruder were repeatable across the laying period. A male's propensity to attack the specimen was repeatable, but other behaviors and our aggression score were not repeatable. Other studies have found that western bluebird aggressive behavior is repeatable throughout the breeding season (Duckworth 2006). Duckworth (2006, and later) used a behavior scoring system that relied heavily on the number of attack behaviors, so our results are in agreement with these earlier studies. Our data suggest that signal honesty of blue coloration may be maintained in this case through behaviors that are likely to result in injury, but "bluffing" behaviors such as aggressive flights, hovers, or nest box defense are not contributing to the

honesty of this signal, as they are not repeatable aspects of male phenotype. However, the fact that more elaborate males are still more likely to engage in the behaviors that escalate contests is compelling, and warrants further study about the maintenance of signal honesty in this system. In great-tits there is strong between-year repeatability of the plastic decline in aggressive behavior throughout the breeding period (Araya-Ajoy and Dingemanse 2017). In our study system, an aggregate of aggressive behavior is not repeatable during a single breeding stage (egg lay), but may be repeatable across years or breeding stages within the same year. We saw no decline in aggression throughout the laying period, and did not perform our intrusions at any point after nestlings had hatched. Sampling individuals in multiple years and across stages of the breeding season will help us understand whether our observed effects are due to selection or a different source of variation.

In conclusion, we have demonstrated that mountain bluebird coloration is related to several indicators of aggressive behavior and competitive ability. Females mated to bluer males receive resource benefits through increased territory quality, and bluer males show evidence of increased nest defense throughout the nestling period. However, only specific aspects of male aggressive behavior are repeatable throughout the laying period. Our results underline the importance of studying multiple possible benefits associated with signal traits. Signals that are relevant in aggressive contexts can also indicate benefits to females, though the relationships are likely to be multifaceted and vary across systems.

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Tables and Table Legends

Behavior	PC1 Loading
Time spent within 10m of the nest box	0.51
Hovers	0.44
Perch on Nestbox	0.32
Attacks	0.37
Aggressive Flights	0.55

Table 1: Loadings of aggressive behaviors on first principal component (PC1)

Figures

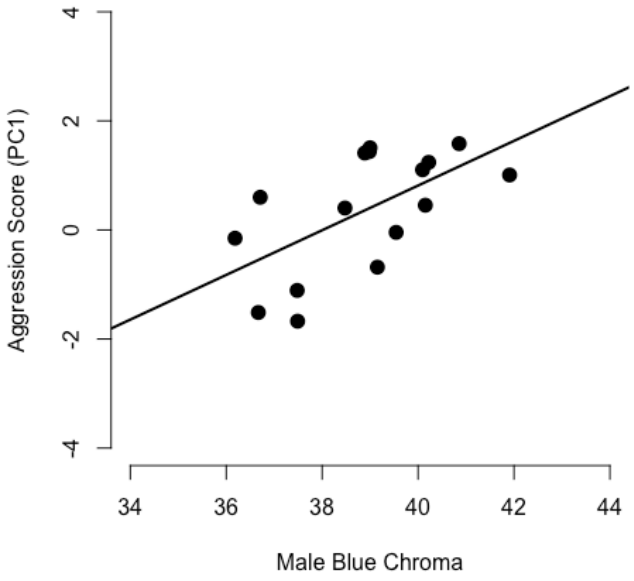

Figure 1 The relationship between focal male coloration and aggression score

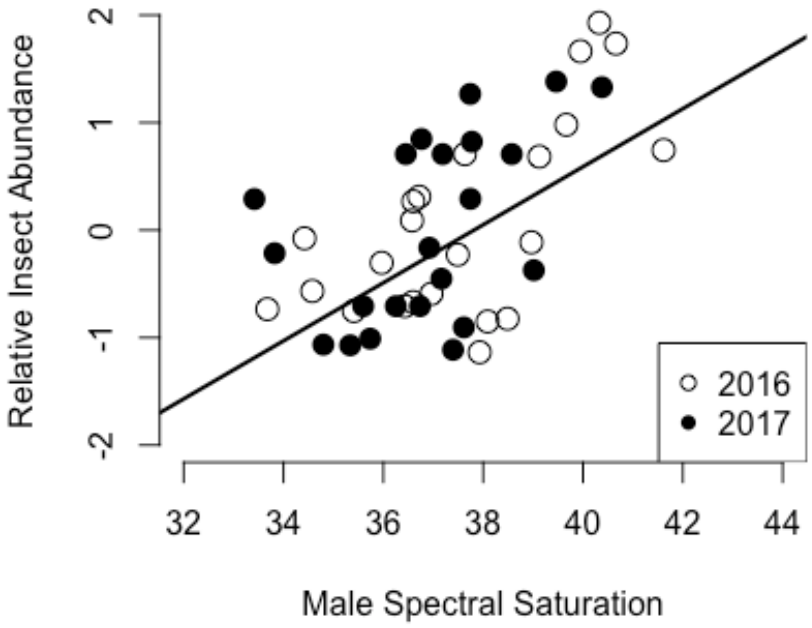

Figure 2 Male coloration predicted his territory quality during 2016 and 2017

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Blue structural coloration honestly indicates male physiological condition under resource limitation in the mountain bluebird, *Sialia currucoides*

Berk, S.A.[1], Emlen, D.J.[1], and Breuner, C.W.[1,2]

[1] Division of Biological Sciences, University of Montana, Missoula, MT, U.S.A.

Abstract

Sexually selected signals are predicted to exhibit heightened condition-sensitive expression relative to other, non-signaling structures. We used corticosterone (CORT) physiology to study the effects of food availability on blue coloration (a sexually selected signal) and feather aerodynamic performance in the mountain bluebird. Stressors as diverse as nutrient limitation, disease, and low social status all increase levels of circulating CORT, making CORT an informative proxy for assessing the overall state/condition of individual males. We found that when birds were food limited, CORT inversely predicted coloration; this relationship disappeared when birds were given ad lib food. Neither food limitation nor CORT affected feather performance, which was unrelated to signaling. To understand these differences in condition-dependence, we also measured feather fine-structural morphology. We found that a component of feather structure (barbule density) negatively predicted coloration and was exquisitely sensitive to CORT, but only when resources were limiting. In contrast, an adjacent feather structural component associated with feather performance (interbarb distance) was not sensitive to CORT and did not predict feather coloration. Our results reveal an astonishing uncoupling of the development of adjacent aspects of the same structure, and provide compelling evidence for the importance of heightened condition-sensitive expression of sexually selected signals.

Introduction

Sexual selection acts on traits that increase mating success, either through female preference or success during competition for access to mates [1]. Classic theory on sexual selection proposes several unifying characteristics of sexually selected traits that increase their reliability as signals of quality to conspecifics [rev. by 2–5]. First, high inter-individual variation in sexually selected traits allows these traits to reveal variation in genetic quality [6–10]. Second, traits favored by sexual selection are often highly condition sensitive, especially compared to non-sexual traits [6,11–15]. For example, rhinoceros beetle horns are more sensitive to variation in larval nutrition than wings or genitals [16,17]. Also, stalk-eyed fly eyestalks reveal sensitivity of different genotypes to variation in nutritional condition, while variation in body size does not [18].

To test these attributes of sexually selected traits, we must first choose an accurate measurement of individual condition [13]. Some have defined condition as the amount of resources an individual is able to devote to the development of a sexually selected trait [19–21]. Others have defined condition as the number of offspring produced relative to other individuals in the population [3,22]**.** Finally, recent theoretical work has proposed that condition represents the ability to respond to environmental challenge [23]. Even under these definitions, many traits could conceivably be related to condition, and researchers who perform empirical work often find it difficult to select traits that represent condition in their study organism. Endocrine systems are a good proxy for individual state/condition because hormones both respond to environmental conditions and regulate internal response and resulting phenotype [24,25]. The hormone corticosterone (CORT) is released by the adrenal glands in response to challenge to divert resources towards self-preservation [26]. CORT secretion is often thought to represent condition and allostatic state [25,27,28]. A rapid, transient increase in CORT can help individuals to survive challenging situations. However, prolonged elevation of CORT can cause damage to other physiological systems and potentially decrease fitness [29,30]. For example, CORT-implanted white crowned sparrows abandon their high-elevation breeding territories and retreat to low elevations during storms [31] Increased CORT in this population predicts increased survival but decreased reproductive success [32]. In terms of sexual selection, CORT-implanted nestling barn owls show reduced investment in a sexually selected trait: deposition of phaeomelanin into their feathers [33]. These links between environmental conditions and potential fitness consequences make CORT an ideal regulator of condition-dependent sexually selected traits.

We performed an experiment to test for heightened condition-dependence of blue coloration in the mountain bluebird, *Sialia currucoides.* We used a resource limitation challenge to explore changes in CORT, feather coloration, and feather aerodynamic performance in response to environmental challenge. While it is common to test condition-dependence of sexually selected traits through resource limitation, many studies do not compare changes in sexually selected traits to non-sexual controls [14]. Therefore, we explored whether blue coloration (a sexually selected trait) was related to feather performance (a non sexual trait), and used the direction and strength of these relationships

to understand the honesty of male coloration. We also investigated whether changes in trait quality across resource levels were related to CORT physiology, and whether this condition-sensitivity occurred when resources were limiting, fully available, or both. Individuals may be able to allocate resources to all traits equally when resources are abundant, but limiting resources may reveal trade-offs between competing demands [34–36]. For example, horn length in soay sheep is negatively associated with longevity only when environmental quality is poor, but the trade-off is absent in high resource years [37].

We explored the relationships between CORT physiology, feather coloration and aerodynamic function, to explore the mechanisms underlying condition dependence of blue coloration. First, feather coloration may be negatively impacted by low food and elevated CORT, but feather performance may be insensitive to both. This would add to results in rhinoceros beetles and stalk-eyed flies demonstrating heightened condition sensitivity of sexual traits compared to non-sexual traits [14,16,17]. Under this hypothesis, we predict that differences in the quality of males should become exacerbated in our resource limitation treatment, resulting in greater variance in CORT responses (reflecting greater variation in male quality), as well as amplified among-male variation in the signal trait (coloration). In contrast, despite the amplified variation in physiology, feather performance should be less affected by food limitation or related to individual CORT secretion. This hypothesis predicts that flight performance will be less sensitive to fluctuations in male physiological condition, and we predict similarly low patterns of among-male variation for this trait across both high and low food availability treatments.

Alternatively, blue coloration may be pleiotropically linked to feather performance. While pigments produce some avian colors, blue coloration results from the reflection of light through highly organized tissues within the feather. Light passes through the feather cortex and is scattered through a spongy layer of organized keratin before reaching a basal layer of melanin granules that reflects the observed color [38,39]. Hereafter, we collectively refer to these structures as "microstructure." In contrast to microstructure, feather macro-structure includes larger components of feather structure such as barbule density, rachis thickness, and mass. Feather macrostructure plays a role in flight performance and thermoregulation [40,41] Because both color and flight performance result from structural morphology of the feather, it is possible that changes in one

necessarily result in changes to the other. If coloration and flight performance are structurally or otherwise pleiotropically linked, then both traits should covary with individual male condition and CORT physiology. In this case, birds would not be capable of uncoupling the expression of color and flight performance, and both traits should be equally condition dependent.

Previous research has explored singular connections between CORT, feather macrostructure, feather color, and feather performance; we sought to understand the connections across physiology, feather color, and feather function within the same species. In European starlings (*Sturnus vulgaris*) CORT affects some, but not all, components of feather macrostructure [42]. That study, however, did not relate feather macrostructure to coloration or performance. Others have evaluated the relationship between feather structure and performance across gross levels of morphology, such as between species, age classes, or feather regions [40,41,43,44], but to date no studies have linked individual variation in feather macrostructure to feather function. Examining feather function in this manner allows us to understand which components of feather macrostructure may relate to feather color, and whether or not these same feather metrics were important for feather function.

Methods

Animals and Housing

In summer 2016, we transported 14 male mountain bluebird nestlings, between 15 and 18 days post-hatch, to our laboratory at the Field Research Station at Fort Missoula. To acclimate nestlings to the laboratory environment, we hand fed nestlings 1 mL of Formula for Nestling Songbirds diet [45] per hour and weaned birds to an adult diet (peanut butter crumble diet, adapted from [45]) as they were ready; see **Appendix 1** for complete hand-rearing protocol. During hand feeding, birds were housed in cages with 2-3 individuals per cage (30in x 18in x 18in). We initially exposed birds to 15-hour day lengths and decreased day length by 15 minutes per week until photoperiod reached 12 hours of light/dark per day. We maintained this 12 hour light/dark cycle for the duration of the experiment. This reduction in photoperiod was sufficient to stimulate birds to molt into their adult plumage. After weaning, birds were released into a flight room (2.5m x 2.5m x 2.5m) to complete the

photoperiod adjustment. We returned birds to individual cages at the beginning of the experiment, and allowed for a one week acclimation to cages before beginning blood sampling. Birds received ad lib water for the duration of their time in captivity, regardless of if they were food restricted. All procedures were approved under permits from the US Fish and Wildlife Service (23228), Montana Fish Wildlife and Parks (2016-078), and the University of Montana Institutional Animal Care and Use Committee (AUP 33-14CBDBS-061014).

Experimental Procedure

We tested the interaction between treatment, feather structure, and corticosterone on feather coloration and performance. To this end, we used a paired study design, where each of the n=14 birds received both the control (ad lib food) and the experimental (20% food reduction) treatment in randomized order. We weighed each individual's food daily before the experiment began to determine average total food intake and then reduced each bird's food intake accordingly. We stimulated feather growth by pulling the two outermost primaries on each wing, the four outermost rectrices, and a large patch of contour feathers from the rump. We pulled these feathers on the first day of the experiment, and allowed birds to grow feathers for 56 days while receiving their designated treatment. We observed variation in the amount of feather regrowth during this time, but 56 days was sufficient for all birds to fully grow at least one remige and all pulled contour feathers. While we pulled feathers from several regions to stimulate heavy molt, we only report results from contour feathers, as field studies have found that rump coloration is positively related to reproductive success [46].

We collected blood samples from birds receiving food limitation or ad lib food to determine baseline CORT secretion 3 weeks into each treatment, resulting in two measurements of baseline CORT per bird. Five samples were lost during processing and 1 was omitted as a statistical outlier, resulting in a final n of 11 for food limitation and 10 for ad lib treatments. During the food limitation treatment, n=7 birds received a CORT implant along with a reduction in available food. However, our CORT implant pellet (Innovative Research of America, SG-111) failed to produce an elevation in CORT levels (**Appendix 2**). We therefore combined treatment groups (cort- and control-implanted) and compared

feather traits against endogenous CORT levels measured in the middle of feather growth for each individual.

Feather Structure and Color

We measured color using a USB4000 spectrophotometer with a pulsed xenon light source (Ocean Optics, Dunedin, USA). We took five reflectance measurements each consisting of ten averaged curves. We stacked seven contour feathers on top of each other and taped them to non-reflective black paper (Canson) for measurements. We positioned the probe at 90 degrees using a probe holder and standardized the distance between the probe and the specimen at 5mm. We standardized measurements between individuals using a white standard, and turned off the light source and covered the probe to create a dark standard.

To extract color variables, we averaged the resultant reflectance measurements (between 300 and 700nm) and smoothed spikes from curves using the program CLR 5 (v. 1.05, Montgomerie 2008). From these averaged curves we used R (R Core Team 2017) to extract the hue (wavelength of peak reflectance), blue chroma (saturation: proportion of the reflectance concentrated from 400-512 nm), UV-chroma (proportion of the reflectance concentrated from 300-400nm), and brightness (sum of the total reflectance). To ease interpretation of our effect sizes, we report chroma variables as whole numbers rather than proportions (proportion reflectance within specified wavelengths x 100).

We evaluated feather structure by measuring barbule density per 1 mm^2, rachis thickness, the distance between individual barbs, and the angle of the barb to the rachis as per [42]. We took two 50X images per feather using the cellSens software package on an Olympus SZX16 fluorescence dissecting microscope with an Olympus DP26 camera attachment (Olympus Corporation, Japan). We collected one image from the tip of the feather, and a second towards the middle of the feather, closer to the proximal end, but not including any downy parts. From these images, we used ImageJ (NIH) to count barbules within two separate randomly selected fields 1 mm^2 near the tip of the feather. We measured rachis thickness, interbarb distance, and barb angle as the average of five measurements taken from the middle of feather. We also measured feather length to the nearest millimeter and feather mass to the nearest milligram.

Feather Resistance to Airflow

We measured feather resistance as the back pressure feathers generated as air was directed through them at a constant rate. Briefly, individual feathers were centered and superglued to 5-mm diameter plastic tubing (8 cm lengths). Air was directed through the feather from a cylinder of compressed air, with the flow rate (100 ml/min) controlled by a mass-flow controller and associated electronics (MFC-4, Sable Systems). We measured the pressure differential across the feather by connecting a tube from a t-junction located just upstream of the feather to a differential pressure meter (PT1000-B, Sable Systems). Between measurements, pressure arising from the t-junction and tubing alone was zeroed out using empty plastic tubing. We measured three feathers per individual per treatment and averaged them before analysis.

Hormone Assays

We measured corticosterone using an enzyme-linked immunosorbent assay (ELISA) kit from Enzo Life Sciences (Cat No. ASI-900-097). Pooled plasma was extracted with diethyl ether and checked for parallelism against the standard curve; all dilutions used in the assay occurred in the parallel portion of the curve. Individual samples were doubly-extracted with diethyl ether according to [47], and run in triplicate at a final dilution of 1:10-1:36 in assay buffer included with the Enzo Life Sciences ELISA kit. Sample recovery was estimated by adding 50 μl of 4000 cpm/100 μl 3H-CORT prior to extraction (mean recovery=76% ± 8%), and assay results were adjusted based on individual recovery values. We analyzed most samples in triplicate during the ELISA, but we often included duplicates to manage space on plates. Intra-assay variation was 7.7% and inter-assay variation (based on an external standard included on each plate) was 14% across 6 plates.

Statistics

We performed all statistical analyses using R Version 3.4.3 [48]. We first examined variables for normality and performed log-transformations where appropriate. We performed paired t tests to determine the effect of our food limitation treatment on mean

corticosterone, feather color, and feather performance. We also used Bartlett's test to examine changes in variance across control and experimental treatments. To test for relationships between CORT physiology, feather color, and structure, we used linear mixed effects models (package nlme) with a random effect of individual ID.

When examining the relationships between feather morphology, coloration, and performance, we sought to limit post-hoc comparisons and preserve degrees of freedom. Therefore, we began our analyses using backwards model selection to determine which feather structure variables were related to feather coloration and performance (Results in sections b and c below). We then used the best models from these analyses (determined by AICc comparisons) to explore interactive effects of treatment and corticosterone on the aspects of feather structure that were relevant to feather color or performance (section d). We chose this approach because we measured many components of feather structure, and this analysis allowed us to use only the feather structure variables which were relevant to feather color or performance and avoid overfitting our models given our small sample sizes. We used generalized linear mixed models with a random effect of individual ID for these analyses. In certain cases, we used linear models within treatment groups (ad lib and food limited) to further explore relationships and determine if overall correlations were equally strong in both groups. As these models did not include repeated sampling of individuals, we did not include any random effect structure. Within our dataset, blue chroma and UV chroma were highly correlated (Pearson r = (0.72, 0.93), $p<0.001$). To simplify our analyses, we only report model selection for structural predictors of blue chroma, though these results never conflicted during model selection of feather structure and UV chroma. We chose to report our results as 95% confidence intervals surrounding estimates of effect sizes.

Results

a) Condition Sensitivity of Feather Coloration and Performance

Food limitation elevated baseline CORT and led to increased variance in baseline CORT among males (**Figure 1a,** paired t test: t=-2.34, df=7, p=0.05, Bartlett's K^2=5.42, p=0.02). Food limitation also increased variation in blue chroma (**Figure 1b,** Bartlett's K^2, Blue Chroma: 4.25, p=0.04) and decreased variation in hue (Bartlett's K^2=11.25, $p<0.01$),

but did not alter variance in brightness (Bartlett's K^2= 1.06, p=0.30). There were no changes in the mean of any of our coloration measures across treatments (paired t tests, Blue Chroma: t=1.20, df =13, p=0.25; Hue: t=1.16, df=13, p=0.27; Brightness: t=-0.09, df=13, p=0.92). Finally, there was no change in the mean or variance of feather performance across treatments (**Figure 1c,** paired t test: t=1.10, df=13, p=0.30, Bartlett's K^2= 1.30, p=0.25).

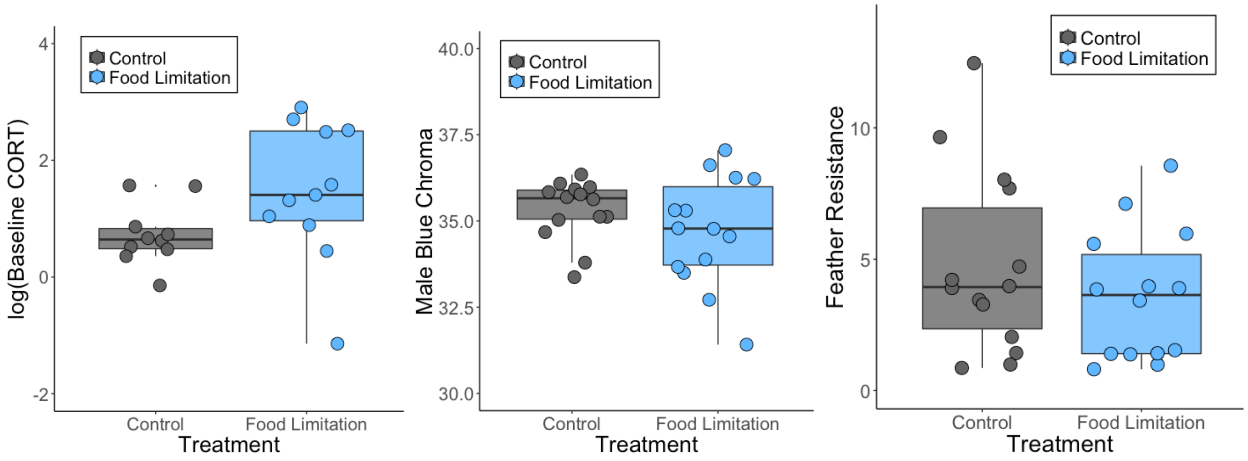

Figure 1 Food limitation increased baseline CORT (a), and increased variance in both CORT and blue chroma (b), but had no effect on feather resistance (c).

We found that CORT predicted coloration, but not feather performance (**Figure 2**). Furthermore, CORT was only related to feather coloration in our resource limitation treatment. In our full mixed-effects model of the relationship between baseline CORT and blue chroma, we found no support for an interaction between treatment and CORT ($\boldsymbol{\beta}_{CORT*Treatment}$ 95% CI= (-2.56,1.20), t_5=-0.92, p=0.39). Our simplified additive model revealed an overall negative relationship between baseline CORT and blue chroma, but no effect of treatment, and performed better than the full model based on AICc ($\boldsymbol{\beta}_{CORT}$ 95% CI= (-1.59,-0.34), t_6=-3.78, p<0.01, $\boldsymbol{\beta}_{treatment}$ 95% CI= (-0.97,1.31), t_6=0.36, p=0.73, ΔAICc = -1.93). Simple linear models within treatments determined that this effect was entirely due to the negative correlation within the food limited group, where the variance in baseline CORT and coloration were greater (Food limited: $\boldsymbol{\beta}_{CORT}$ 95% CI = (-1.79, -0.311), $F_{1,9}$=10.3,

p=0.01, Ad lib: $\boldsymbol{\beta}_{CORT}$ 95% CI = (-1.56,0.90), $F_{1,8}$= 0.38, p=0.55). However, the large overlap in confidence intervals for the effect sizes suggests that while the effect of corticosterone on coloration is driven by changes in variance within the food limited group, the effect is not necessarily different from birds in the ad lib group. In contrast, we found that CORT did not predict variation in feather resistance in either treatment (LMM: $\boldsymbol{\beta}_{CORT*Treatment}$ 95% CI= (-3.88,7.53), t_5= 0.82, p=0.45, additive LMM: ($\boldsymbol{\beta}_{CORT}$ 95% CI = (-0.94,2.85), t_6=1.22, p=0.27, $\boldsymbol{\beta}_{Treatment}$ 95% CI = (-6.01,1.22), t_6=-1.62, p=0.15).

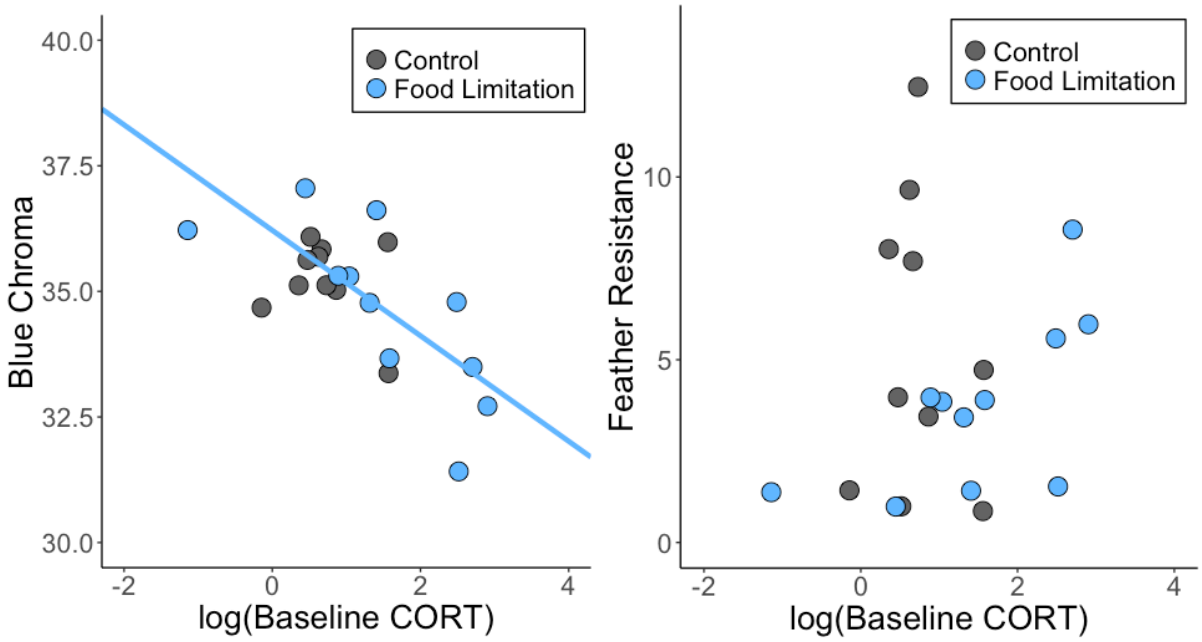

Figure 2 Baseline CORT predicts blue chroma (left panel), but not feather resistance (right panel).

b) Feather Coloration and Structure

Feather macrostructure and coloration co-varied depending on treatment. Our model selection revealed that while our best model included effects of barbule density, barbule density was not a good predictor of feather coloration across treatments. (LMM: $\boldsymbol{\beta}_{barbule}$ 95% CI= (-0.01, 0.006), $\boldsymbol{\beta}_{Food\ limited}$ 95% CI= (-1.73, 0.475), **Table S1**). Instead, barbule density and blue chroma were negatively correlated within the food limited treatment, but not in the ad lib treatment (**Figure 3,** Ad lib: **β** 95% CI= (-0.004, 0.01), $F_{1,11}$=1.26, p=0.28, Food limited: **β** 95% CI= (-0.03, 0), $F_{1,12}$=4.48, p=0.05)).

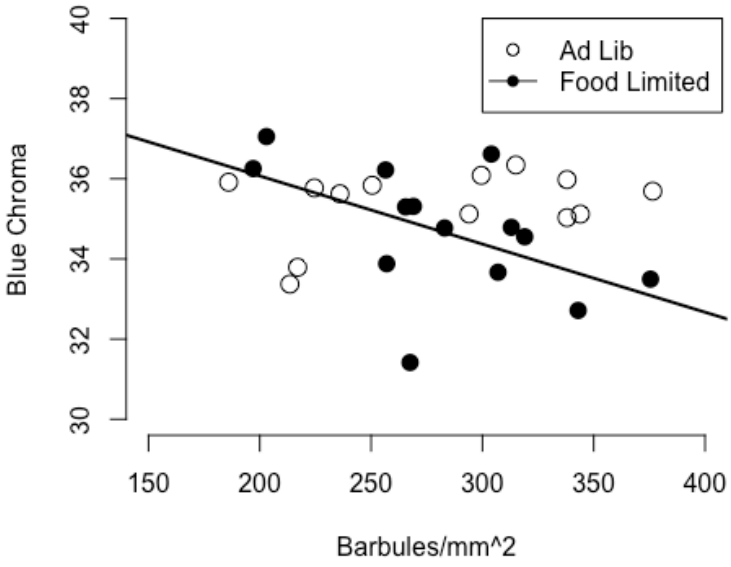

Figure 3 Barbule density and blue chroma are negatively related under food limitation (filled circles) but not ad lib food availability (open circles).

c) Feather Performance and Structure

Specific feather macrostructure variables predicted feather performance. In our top model, interbarb distance was negatively correlated with feather resistance (**Figure 4,** LMM: $\mathbf{B}_{\text{Interbarb}}$ 95% CI **=** (-57.69,-8.02)), and food limitation had no effect on feather resistance (LMM: $\boldsymbol{\beta}_{\text{Treatment}}$ 95% CI **=** (-4.24,0.11)). While this model was equivalent to our model including both barb angle and rachis thickness (**ΔAICc** = -0.37), these two variables were ultimately unrelated to feather resistance ($\boldsymbol{\beta}_{\text{BarbAngle}}$ 95% CI **=** (-0.26,0.45), $\boldsymbol{\beta}_{\text{Rachis}}$ 95% CI **=** (-66.97, 44.28)). We did not include feather mass in our model selection due to colinearity with other components of feather structure, but feather mass and resistance were significantly positively correlated (**β** 95% CI =(6.11,11.77)). Our model selection results for the effects of feather structure on resistance to air are summarized in **Table S2**.

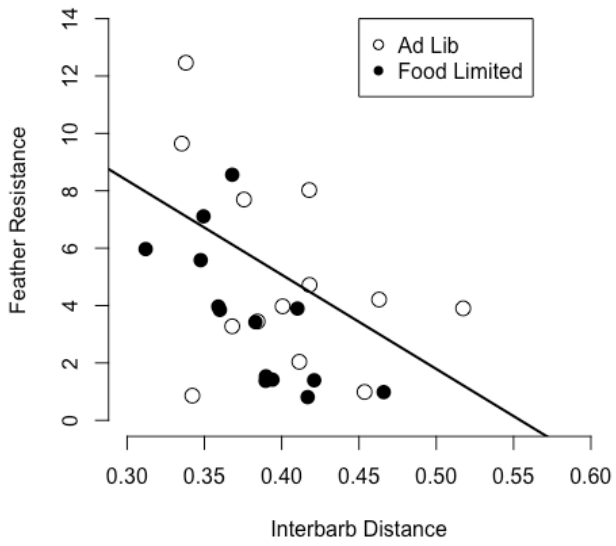

Figure 4 Interbarb distance predicts feather resistance across ad lib (open circles) and food limitation (filled circles) treatments.

d) Corticosterone and Feather Structure

We used our analyses from parts (b) and (c) to drive our analysis of the effect of corticosterone on feather structure. Hence, we only evaluated CORT's association with interbarb distance, barbule density, and feather mass.

Corticosterone had varying effects on feather macrostructure across treatments. Our top model for the effect of corticosterone on barbule density included the interaction of corticosterone and treatment, though it was not significant ($\boldsymbol{\beta}_{CORT*Trt}$95% CI **=** (-55.81, 159.96)). However, analyses of the treatment groups separately revealed that birds in the food limited treatment increased barbule density with increasing corticosterone while birds in the ad libitum group did not (**Figure 5**, Ad lib: $\boldsymbol{\beta}_{CORT}$ 95% CI **=** (-133.73, 83.71) $F_{1,7}$= 0.30, p=0.60; Food limited: $\boldsymbol{\beta}_{CORT}$ 95% CI **=** (5.97,48.15), $F_{1,9}$= 8.42, p=0.02).

We found no significant relationship between corticosterone and interbarb distance. Our best model for the effect of corticosterone on interbarb distance included only an effect of corticosterone, though it was not significant ($\boldsymbol{\beta}_{CORT}$**=** (-0.03, 0.006)). Individual models revealed no effect of corticosterone on interbarb distance in either treatment (Ad lib: $\boldsymbol{\beta}_{CORT}$ 95% CI **=** (-0.10,0.06) $F_{1,7}$= 0.361, p=0.56, Food limited: $\boldsymbol{\beta}_{CORT}$ 95% CI **=** (-0.03,0.005), $F_{1,9}$=

2.75, p=0.13). We summarize these model selection results in **Table S3**. Hence, corticosterone predicts changes in traits associated with color, but not feather performance.

We found no significant relationship between corticosterone and feather mass. Our best model for the effect of corticosterone on feather mass included only an effect of treatment, though it was not significant ($\boldsymbol{\beta}_{\text{Treatment}}$ 95% CI = (-0.36,0.06)).

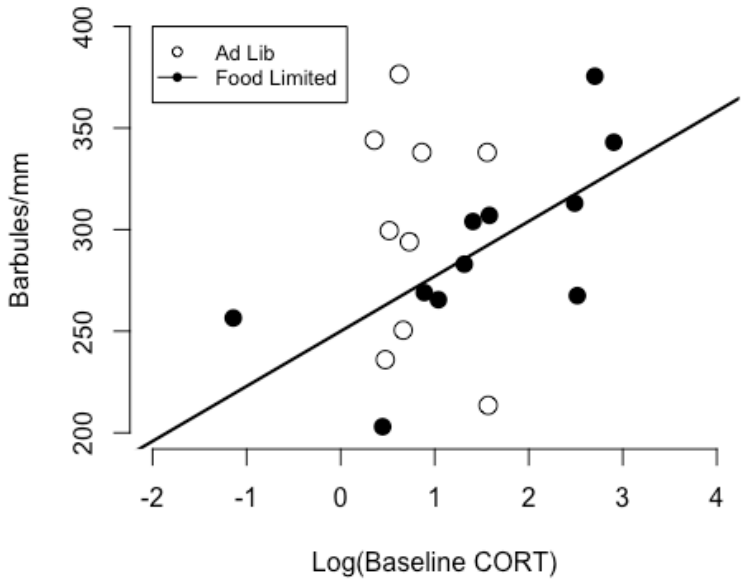

Figure 5 Baseline CORT predicts barbule density in the food limited (closed circles), but not the ad lib (oopen circles) treatment.

Discussion

Our results demonstrate that the sexually selected functions of feathers (coloration) are sensitive to condition, while the naturally selected functions (resistance to airflow) are not. We found that these differences were predicted by variation in individual physiology in response to resource limitation. Namely, males that secreted more CORT in response to our experimental challenge sacrificed color production. However, the same was not true for the naturally selected components of feathers that regulated feather function.

Signaling theory predicts that traits functioning as reliable indicators of individual male quality in the context of sexual selection should be more sensitive to stress, nutrition, and variation in individual condition, than other non-signal structures [1,5,49,50]. Indeed, heightened conditional expression amplifies otherwise-subtle differences among males, causing these traits to be especially informative when used as signals in the context of male-male competition or mate choice. Although many studies have demonstrated condition- and/or nutrition-sensitivity of sexually selected signal traits, relatively few have compared these responses to those of other, non-signal traits. Rhinoceros beetle horns are more sensitive to nutrition, and to insulin receptor knockdown during development, than genitals or wings [16,17]. In stalk-eyed flies, eyestalk length reveals genetic variation in responsiveness to resource limitation more strongly than wing length, which scales with body size regardless of resource availability [18]. In our study, blue coloration and barbule density were exquisitely sensitive to male condition, and differences between males became increasingly pronounced as resources became limiting.

It is important to note that the effects of CORT and resource limitation occurred without changes in mean coloration or feather structure across treatment groups. In birds, past work has sought to demonstrate condition dependence of ornamental feather traits through mean decreases during resource restriction or immune challenge [51–55]. While these large differences between treatment groups are one method of demonstrating condition dependence, our results show that individual variation in response to resource limitation is another important facet of honest signaling. Others have previously proposed that sexually selected traits signal individual capacities to respond [23]. Under this hypothesis, a male's ability to cope with challenge is a crucial component of fitness that is expressed through development of sexually selected traits. Our results suggest a novel method of evaluating this hypothesis through the exploration of individual variation in stress physiology in resource-abundant and resource-limited conditions.

Our results confirm critical predictions of sexual selection and signaling theory and show how even subtle aspects of structure on the same feathers can exhibit markedly different patterns of condition- and nutrition-dependence. However, we found that the correlation between barbule density and coloration was inconsistent across resource levels. Past researchers have reported correlations between feather micro- and

macrostructure and color [56,57], and [39] found that individual variation in feather microstructure was related to UV-violet chroma and spectral saturation. Namely, male eastern bluebirds with more circular keratin rods within the feather barb and less variation in keratin rod diameter displayed colors that were more saturated [39]. While these microstructural elements were strongly predictive of feather coloration, others have also observed that macrostructure contributes to feather coloration by altering structural absorption of light wavelengths [57]. We suggest that interactions between micro- and macrostructural components of feathers were altered during our food limitation, which led to stronger associations between barbule density and coloration when resources were limiting. Future work should explore which aspects of feather morphology are related to coloration, and in what environmental contexts.

Our study is unique in that it explores individual variation in the relationship between feather macrostructure and performance. While we frequently measure aspects of feather structure, few studies quantify how individual differences in feather structure are related to feather performance [42,43]. We found that interbarb distance affected feather performance in both treatments, but was not sensitive to CORT physiology. Others have demonstrated that feather structure is relevant to feather function, namely that throughout ontogenetic development, barbule density and rachis thickness are important for age-related increases in flight performance [40]. Feather structure is also relevant to life-history differences between species, as birds with shorter nestling periods develop feathers with less densely-packed barbs [43]. Here, we report that individual variation in feather structure predicts feather resistance, linking structure to performance for the first time.

Overall, our results lend support to condition dependence of blue coloration through interactions with feather macrostructure, stress physiology, and resource availability. However, we were unable to evaluate several interesting components of this system that are worthy of further study. First, our results cannot demonstrate that CORT levels drive the variation we see in food limited individuals. Successful experimental alteration of CORT would be necessary for that, and warrants future work. Future studies could focus on the components of feather development within the follicle to understand when and how differential sensitivity to CORT may occur. Also, there is likely interplay

between feather micro- and macrostructure, and similar to our results here, these relationships may change as resources become limiting. To fully understand the mechanisms underlying variation in blue coloration, full exploration of condition-dependent changes in microstructure are warranted.

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(doi:10.1038/s41467-017-02088-w)

Supplemental Material

Tables

Model	AICc
Blue Chroma ~ Barbule + Barb Angle + Rachis + Interbarb + Food Limitation	111.62
Blue Chroma ~ Barbule + Barb Angle + Rachis + Food Limitation	113.56
Blue Chroma ~ Barbule + Barb Angle + Food Limitation	116.62
Blue Chroma ~ Barbule + Food Limitation	**110.08**

Table S1 Results of backwards model selection of the relationship between feather macrostructure and coloration, all models include a random effect of individual ID.

Model	AICc
Resistance ~ Barbule + Barb Angle + Rachis + Interbarb + Food Limitation	140.87
Resistance ~ Barb Angle + Rachis + Interbarb + Food Limitation	129.88
Resistance ~ Barb Angle + Interbarb + Food Limitation	134.63
Resistance ~ Interbarb + Food Limitation	**129.51**
Resistance ~ Food Limitation	140.69

Table S2 Results of backwards model selection for the effect of feather structure on performance (resistance to airflow), all models included a random effect of individual ID.

Model	AICc
Barbule Density	
Barbule ~ CORT* Food Limitation	**194.54**
Barbule ~ CORT + Food Limitation	201.29
Barbule ~ CORT	206.81
Barbule ~ Food Limitation	207.63
Feather Mass	
Mass ~ CORT* Food Limitation	26.62
Mass ~ CORT + Food Limitation	22.42
Mass ~ CORT	17.69
Mass ~ Food Limitation	**15.22**
Interbarb Distance	
Interbarb ~ CORT * Food Limitation	-31.92
Interbarb ~ CORT + Food Limitation	-41.11
Interbarb ~ CORT	**-50.83**
Interbarb ~ Food Limitation	-49.37

Table S3 Results of backwards model selection for the effect of corticosterone on candidate feather structure variables. All models include a random effect of individual ID.

Appendix One

Hand Feeding Protocol

We collected nestlings between days 15 and 18 post-hatch and transported birds to captivity in small groups of 2 to 5 birds per day. We chose these ages because mountain bluebird nestlings fledge between 19 and 21 days post-hatch, and we wanted nestlings to complete the majority of energetically expensive growth in the wild.

For the first 1-3 days after bringing nestlings into captivity, we fed nestlings the Formula for Nestling Songbirds (FoNS) at one-hour intervals during daylight hours. We did

not perform any feedings at night and there was no mortality during night cycles. We weighed nestlings morning and night to document mass gain or loss during the day, and made adjustments to the feeding schedule for each individual based on mass change during each day. In general, nestlings that gained or maintained weight received less feedings on the following day. We found this approach useful, and only once had to resume feeding more often when a nestling failed to maintain weight as we decreased feeding. Nestlings typically lost weight for the first five days in captivity, and began to gain weight after this point (**Figure 1)**. On average, it took 10.9 ± 0.4 days to wean nestlings onto an adult diet using this protocol (**Figure 2**). Our fastest individuals began eating their adult diet in 9 days, and the slowest took a total of 15 days. We determined that nestlings were eating the adult diet through observations of food dishes and mass changes throughout the hand feeding period.

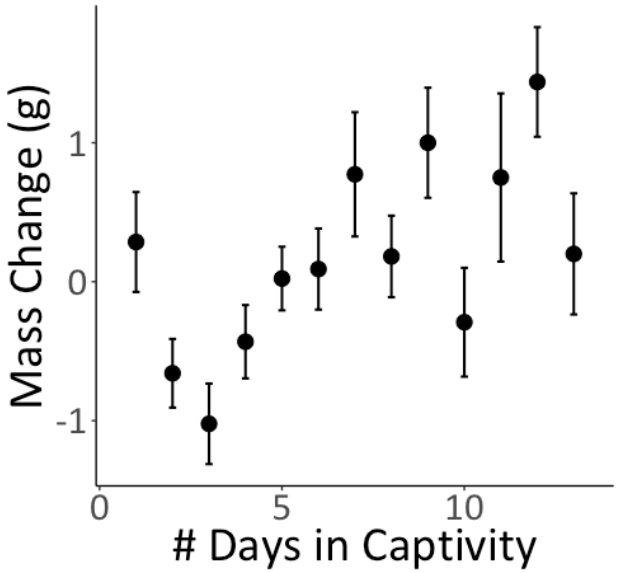

Figure 1 Mass change (mass at end of day – mass at start of day) ± standard error for 22 individual mountain bluebird nestlings during hand feeding.

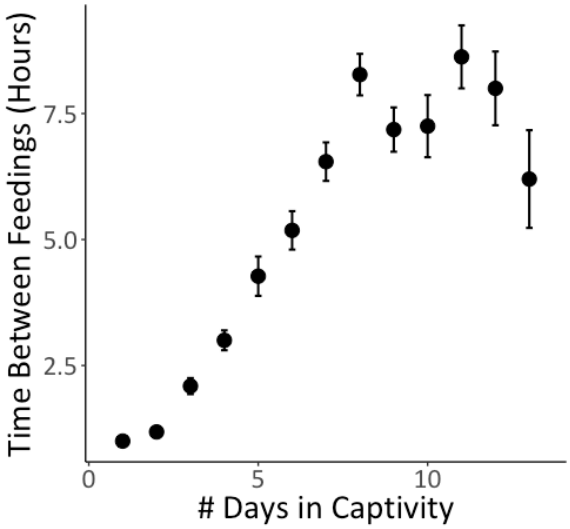

Figure 2 Hours between feedings ± standard error for 22 individual mountain bluebird nestlings during hand feeding.

To entice nestlings to eat our adult diet, we kept fresh dishes of peanut butter crumble (**Table 1,** recipe adapted from Gage and Duerr 2008) available at all times. Approximately every four hours, we added 5-10 mealworms to these dishes. We observed that nestlings cued into the movement of the worms, and subsequently associated the dish with food and began to sample our provided diet as the interval between hand feedings increased. It is important to note that we did not want the mealworms to be a significant or predictable source of food, and they were merely used as a training tool to adjust birds to captivity. Using this protocol, we had no deaths due to starvation.

Ingredient	**Amount**
Toasted wheat germ	2 cups
Hills Science Diet feline maintenance dry food	2 cups, ground
ZuLife Soft-Bill Diet for Iron Sensitive Birds #5MI2	2 cups, ground
Quiko Goldy Eggfood	½ cup
LaFeber Avi-Era bird vitamins	1 tablespoon
Calcium carbonate	1.5 tablespoons
"Old fashioned" peanut butter (no salt, sugar, or other additives)	½ cup

Table 1 Recipe for peanut-butter crumble diet fed to bluebirds as adults. All ingredients were combined in a food processor.

Appendix Two

After we completed our experiment, we selected three birds that had not received a CORT implant as one of their experimental treatments. We gave these individuals corticosterone implants and measured baseline CORT before the implant (**Figure 1,** day 0), 3 day post-implant, and every seven days thereafter for 34 days.

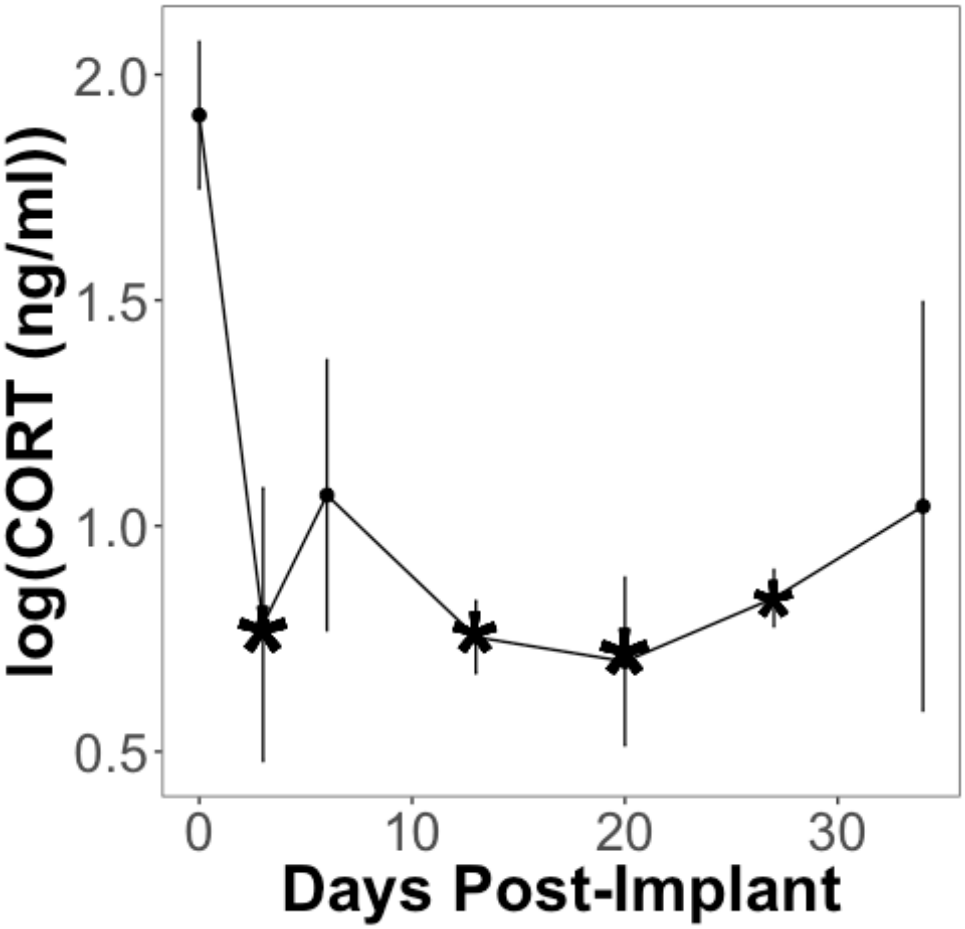

Figure 1 Changes in baseline CORT across time after CORT implantation. Our CORT implant significantly decreased CORT secretion. Stars indicate points that are significantly different from our pre-implant blood sampling.

We used a repeated measures ANOVA to compare CORT levels amongst our sampling days during this period, and performed Tukey comparisons to determine differences between days post-implantation. We found that CORT levels were decreased three days after implantation, increased to normal levels seven days post-implant, and remained depressed until 34 days post-implant, when they returned to pre-implant levels (**Table 1**).

Comparison	Difference ± SE	z	Tukey HSD p
Day 3 – Day 0	-1.12 ± 0.31	-3.59	<0.01
Day 6 – Day 0	-0.85 ± 0.31	-2.67	0.15
Day 13 – Day 0	-1.16 ± 0.31	-3.67	<0.01
Day 20 – Day 0	-1.21 ± 0.31	-3.85	<0.01
Day 27 – Day 0	-1.06 ± 0.31	-3.40	0.01
Day 34 – Day 0	-0.86 ± 0.35	-2.47	0.28
Day 6 – Day 3	0.29 ± 0.31	0.91	1.00
Day 13 – Day 3	-0.03 ± 0.31	-0.08	1.00
Day 20 – Day 3	-0.08 ± 0.31	-0.26	1.00
Day 27 – Day 3	0.06 ± 0.31	0.19	1.00
Day 34 – Day 3	0.26 ± 0.35	0.74	1.00
Day 13 – Day 6	-0.31 ± 0.31	-1.00	1.00
Day 20 – Day 6	-0.47 ± 0.31	-1.17	1.00
Day 27 – Day 6	-0.23 ± 0.31	-0.72	1.00
Day 34 – Day 6	-0.02 ± 0.35	-0.07	1.00
Day 20 – Day 13	-0.05 ± 0.31	-0.17	1.00
Day 27 – Day 13	0.09 ± 0.31	0.28	1.00
Day 34 – Day 13	0.29 ± 0.35	0.83	1.00
Day 27 – Day 20	0.14 ± 0.31	0.45	1.00
Day 34 – Day 20	0.34 ± 0.35	0.98	1.00
Day 34 – Day 27	0.20 ± 0.35	0.58	1.00

Chapter 3: Context-dependent direct benefits across years and seasons in the mountain bluebird, *Sialia currucoides*

Berk, S.A. & Breuner, C.W.

Abstract

Sexual selection is a complex process that results from selection on traits through differential mating success. Sexually selected traits are honest if they are related to individual condition and predict individual performance. For traits that are under selection through female choice, traits may relate to benefits that males provide to females or offspring. We explored whether a sexually selected trait (blue coloration) was a consistently honest predictor of direct benefits in the mountain bluebird, *Sialia currucoides.* We present three years of data concerning variation in the relationship between bluebird coloration and direct benefits to females, expressed as offspring quality (nestling mass). We found that between years and seasonal timing (first vs. second broods), the relationship between male coloration and nestling mass varied from negative to neutral or positive. In some contexts, more elaborate males had heavier nestlings, while in other contexts they raised the lightest nestlings. We found that this variation was due to changes in optimal reproductive effort. When average nestling mass at our study site was higher, bluer males raised heavier nestlings, however when average nestling mass was lower, this trend reversed and bluer males raised lighter nestlings. Overall, these results demonstrate both the performance correlates of blue coloration and how the honesty of this trait varies across environmental contexts.

Introduction

The diversity of sexually selected traits in nature has fascinated researchers for some time. There are several models for the maintenance of female preferences for sexually selected traits (Kokko et al. 2003). In some systems, sexually selected traits indicate benefits that males will provide to females. These benefits can occur directly, or indirectly (Kirkpatrick 1985, 1987, Lande 1981, Andersson 1986). Indirect benefits result

from heritability or both sexually selected traits and mating preferences for those traits. This means that females who mate with high quality males produce offspring that have increased mating success in future generations (Fisher 1930). However, direct benefits are fecundity or resource benefits that females receive as a result of exercising their preference for male traits (Iwasa and Pomiankowski 1999, Kirkpatrick 1996, Kokko et al. 2003, Møller and Jennions 2001)

Many studies have confirmed that sexually selected traits honestly indicate a male's ability to provide direct benefits to females (Møller and Jennions 2001). For example, male house finches with redder plumage provide more parental care (Hill 1991). Furthermore, male sticklebacks with more intense red coloration are better able to defend their young at nesting sites (Andersson 1994). However, there are other examples of negative or neutral correlations between indicator traits and direct benefits. Some populations of house finches display conditional strategies, where males with less developed ornamentation perform more parental care to increase offspring quality, while more ornamented males nest earlier to increase offspring survival without increasing parental care (Badyaev and Hill 2002). Also, female pied flycatchers display preferences for larger male song repertoires, but these males with larger repertoires do not feed their offspring more frequently (Rinden et al. 2000). Some have even observed variation in trait information content across a single season; male collared flycatchers with larger plumage badges fledge fewer offspring early in the season, but this effect is reversed later in the season when males with larger forehead patches fledge more offspring (Qvarnström et al. 2000). While these types of effects have been widely discussed and documented in the literature, we have few examples of the environmental or individual variables that drive variation in the links between sexually selected traits and direct benefits (Kokko et al. 2003, Mays and Hill 2004, Miller and Svensson 2014, Qvarnström and Forsgren 1998).

We examined the potential for variation in direct benefits in the mountain bluebird, *Sialia currucoides.* Mountain bluebirds display sexually dimorphic UV-blue coloration, and males with more intense coloration sire more offspring at their own nest and at other nests through extra-pair fertilizations (Balenger et al. 2008, O'Brien and Dawson 2011). However, it is unclear which mechanisms maintain female preference for this trait. There is ample potential in this system for both direct and indirect benefits, as males provide

parental care and participate in aggressive contests to obtain and defend territories. Furthermore, females select both social mates who provide parental care, and extra-pair mates that provide only genetic material (Balenger et al. 2008). In this study, we focused on social mates, as they, rather than genetic mates, are most likely to provide direct benefits.

Past work has found that males with more saturated coloration are more aggressive during contests for territories (Berk, unpublished), but there is no relationship between male coloration and his provisioning rates to offspring (Balenger et al. 2007). Blue coloration is a sexually selected trait that is highly condition sensitive, and its expression is correlated with individual variation in male CORT physiology, such that males with the lowest CORT responses produce the most saturated coloration (Berk, unpublished). These effects are most pronounced during resource limiting conditions (Berk, unpublished). This means that the extent of among-male variation in plumage, and the relationship between plumage quality and male condition, is likely to be more pronounced during harsh years than in good years. In this study, we explored whether male coloration was related to offspring mass, a direct benefit that represents reproductive effort by both parents, and how the relationship between male coloration and the mass of his offspring varied across years and seasons. Overall, we were interested in the reliability of blue coloration as a signal of direct benefits, given the realities of heterogeneous environments experienced by individual birds across their lifetime.

We used several components of natural environmental variation to evaluate these changes in direct benefits. First, we observed variation in spring phenology across the three years of our study. Second, we observed variation across broods within each season. While food availability is often higher during the late summer, life history trade-offs often dictate that during second broods, clutch sizes are smaller, and nestlings weigh less and grow more slowly (Klomp 1970, Martin 1987, Stearns 1989). We were interested in whether these trade-offs impacted males differently based on the development of their sexually selected trait. Finally, we experimentally challenged males by feather clipping them to 'reduce' environmental food availability. Here, we explored whether male coloration predicted his responsiveness to our experimental challenge. We quantified the amount of variation in the relationship between our sexually selected trait and direct benefits, and explored the factors that caused these disparities in male performance.

Methods

Field Site and Nest Monitoring

We studied mountain bluebirds outside of Ronan, MT on the Flathead Indian Reservation (47.478370, -114.377034) from March 20th to August 30th during 2015, 2016, and 2017. The study site consists of 48 nest boxes spread across seven miles of fence line on a dirt road through sagebrush habitat. We scored nest development on a scale of 1-4 and checked highly developed nests (score 3 or 4) every other day until first egg. We checked nests daily throughout the laying period until we had confirmed the onset of incubation through egg warmth and constant clutch size for three consecutive days. During incubation, we checked nests every three days until the 12th day of incubation, at which point we began to check nests daily until nestlings hatched.

We visited nests to measure nestlings on days 3,6,9, and 12 post-hatch, and thereafter we measured nestlings every other day until fledging, which usually occurred when nestlings were 20 days old. At each time point, we took measurements of nestling head+bill length, wing chord, tarsus, and mass. Mountain bluebird nestlings begin to lose weight as they prepare to fledge, so we calculated the maximum mass that each nestling reached before they began to lose weight.

We captured adult males between day 7 and 12 post hatch using nest box traps. For each male, we measured head+bill, wing chord, tarsus, and mass to the nearest gram. Each male received a USFW leg band and a unique combination of colored leg bands. At this capture, we also collected a feather sample for future coloration measurement.

We used NOAA climate data from Hot Springs, MT (47.6°, -114.68333°) to obtain daily minimum, maximum, and average temperature for our study site. The weather station is 10 miles from our nest boxes.

Feather Clip Manipulation

During first broods in 2016, we captured adult male mountain bluebirds using nest box traps on day 7 or 8 post hatch to perform the feather clip manipulation. We attempted captures at all nests on day 7, and if we captured a male on day 8 we designated that nest as a control (unclipped) nest, such that all males who were feather clipped received the

manipulation on nestling day 7 (n=13 control nests, n=13 clipped nests). We alternated treatment designations so both control and feather clip nests were spaced evenly throughout the season. For our wing area reduction, we reduced wingspan by 10% by clipping the outermost five primary feathers. This reduction usually amounted to 10-15mm clipped from each feather. According to the Rankine-Froude momentum model, this increased the power requirements for bluebird flight by 11%. Three control and one feather clip nest experienced nest failure, and we removed these from our analysis.

Color Measurement

We measured the color of rump feathers collected during capture using a USB4000 spectrophotometer with a pulsed xenon light source (Ocean Optics, Dunedin, USA). We took five reflectance measurements each consisting of ten averaged curves. We stacked seven feathers on top of each other and taped them to non-reflective black paper (Canson) for measurement. We positioned the probe at 90 degrees using a probe holder and standardized the distance between the probe and the specimen at 5mm. We standardized measurements between individuals using a white standard, and turned off the light source and covered the probe to create a dark standard. To minimize variation we measured coloration on a single day each year, such that all males from 2015 were measured on a single day in late-summer 2015, and the same for 2016 and 2017. Past measurements of repeatability of color measurements from the same observer (SB) in our lab indicate low coefficients of variation even when feather samples from a single individual are measured several years later (CV Hue=6%, CV Brightness=9%, CV spectral saturation=4%, CV UV Chroma=4%).

To extract color variables, we averaged the resultant reflectance measurements (between 300 and 700nm) and smoothed spikes from curves using the program CLR 5 (v. 1.05, Montgomerie 2008). From these averaged curves we used R (R Core Team 2017) to extract the hue (wavelength of peak reflectance), spectral saturation (proportion of the reflectance concentrated within 100nm each individual's hue), UV-chroma (proportion of the reflectance concentrated from 300-400nm), and brightness (sum of the total reflectance).

Statistics

We conducted our analyses using R version 4.1.3 (R Development Core Team, 2017). For our analyses of overall relationships between male coloration and direct benefits across years, we made sure to exclude any individuals that had received an experimental manipulation during the current breeding attempt. Our final data set included 215 individual breeding attempts from 117 individual males. Within these nests, we measured nestlings at 133 nests.

We tested for differences in phenology (timing and variance in nest initiation date) as well as changes in the mean and variance of male coloration across years using mixed effects models in R package "nlme" with a Gaussian distribution and a log link function (Pinheiro et al. 2017). Each of these models contained a random effect of male ID, as we captured males several times both within years (once each during first and second broods) or between years as they returned to breed at our study site. We performed posthoc comparisons between years using Tukey's HSD in the package "multcomp" (Hothorn et al. 2008). We tested for variance heterogeneity in nest initiation and coloration using Bartlett's tests.

We also used mixed effects models to examine the overall relationship between coloration and nestling mass across years. However, to estimate individual effect sizes across years and between first and second broods within a year, we used linear regression analyses with no random effects. These regressions did not contain multiple observations of the same individual, as males only had one nest within each time period. We used Pearson's r to estimate the relationship between average nestling mass and the effect size for male coloration within a given time period. We also used fixed effects linear regressions to analyze the interaction between color and treatment during our experimental feather clip challenge, as these experiments did not include repeated observations of any individuals.

Results

Annual Variation in Climate and Phenology

Annual variation in the timing of spring led to variation in first egg date across the three years of our study (**Figure 1**). Average nest initiation date was 10 days earlier in 2016 than it was in 2017 (Mixed effects model: $F_{2,69}=16.51$, $p<0.001$, **Table 1**). Between years, there was also variance heterogeneity, such that when birds initiated their nests earlier in 2016, there was less variance in nest initiation date across the study site. Furthermore, in 2017 when average nest initiation was the latest, variance in lay date also increased (Bartlett's $K^2 = 15.639$, df = 2, $p<0.001$, SD(2015)=6.45, SD(2016)=5.82, SD(2017)=10.82).

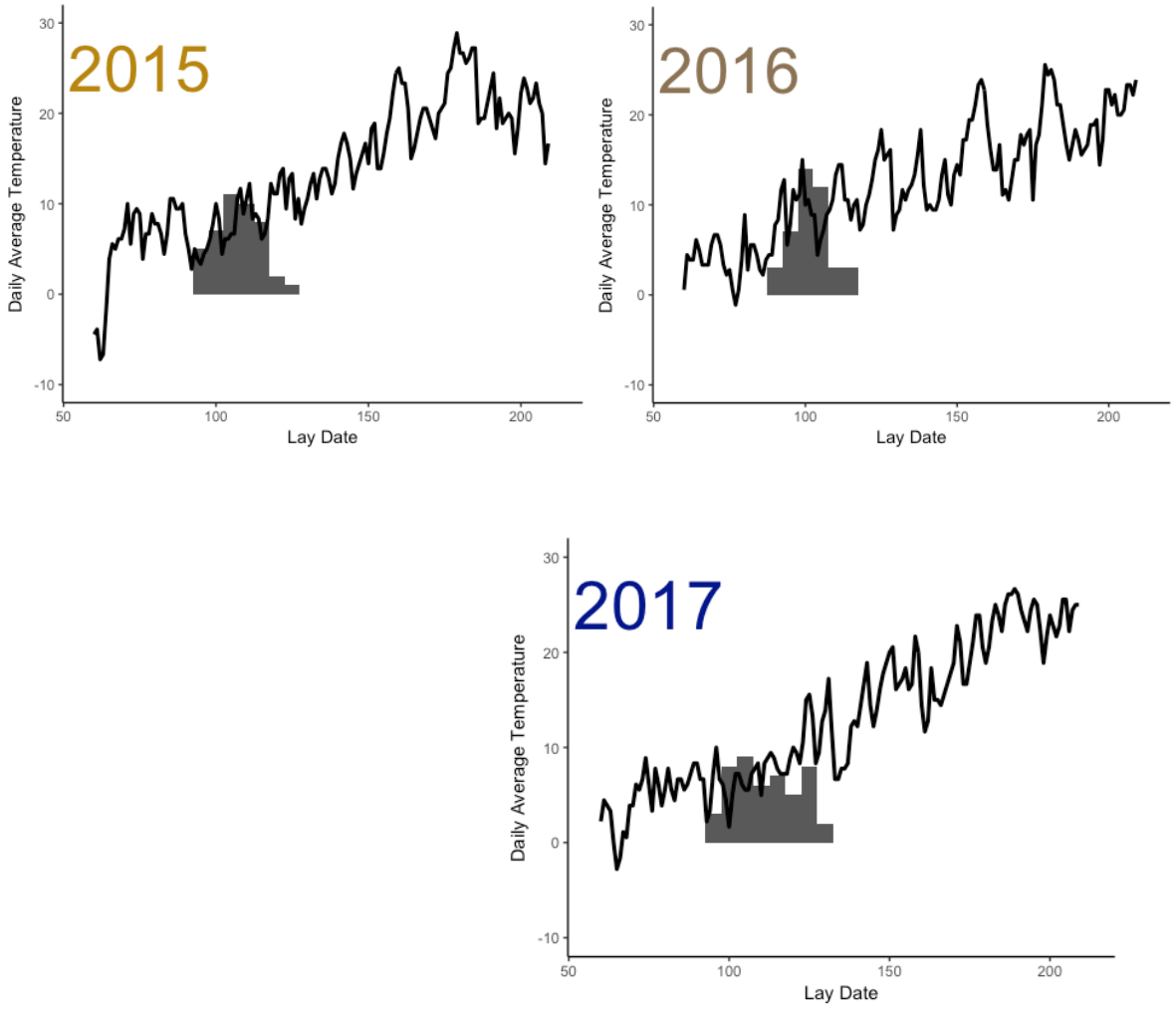

Figure 1 Seasonal variation in temperature and lay date frequencies. Solid lines represent daily average temperature; histograms represent the frequency of nests initiated on each day of the season.

Year Comparison	Difference in Average nest initiation ± SE	p value
2015-2016	5.98 ± 1.80	<0.01
2016-2017	-9.63 ± 1.87	<0.001
2017 - 2015	3.65 ± 1.70	0.15

Table 1 Pairwise comparisons of differences in nest initiation between years with Bonferonni adjusted p values.

Annual Variation in Coloration

We also observed variation in the coloration of males that obtained territories within our study area (**Figure 2**). Males at our study site had less saturated coloration in 2015, there was no significant difference in mean coloration between 2016 and 2017 (mixed effects model: $F_{2,125}$=20.39, p<0.001, mean(2015) = 34.04, n=35 males, mean(2016) = 36.68, n=49 males, mean(2017) = 36.75, n=44 males). Furthermore, we also observed changes in the variance of male coloration across years (Bartlett's K^2 = 5.41, df = 2, p=0.06, SD(2015) = 2.29, SD(2016) = 1.71, SD(2017) = 2.36).

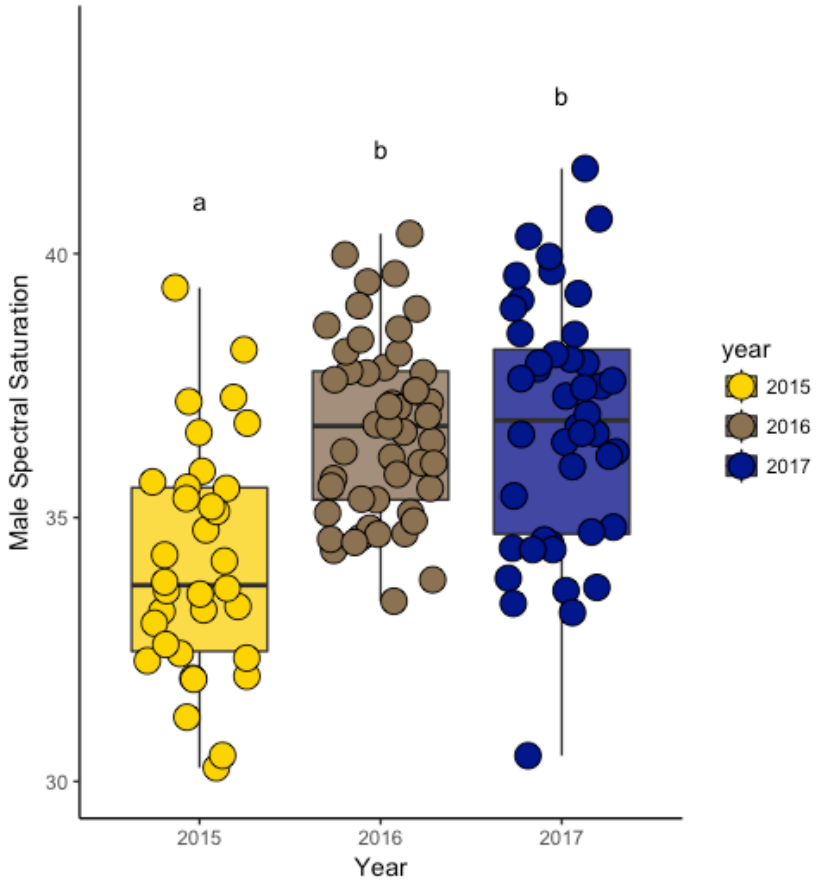

Figure 2 Changes in distribution of male coloration across years. Letters indicate significant differences.

Variation in Direct Benefits

Across years, males with intermediate coloration had the heaviest nestlings (**Figure 3**, β_{Color}=2.49, p=0.05, β_{Color^2}= -0.03, p=0.04). However, within years and across seasons, the relationship between male coloration and nestling mass varied from negative, with more elaborate males having lighter nestlings, to positive, with more elaborate males having heavier nestlings **(Figure 4, Table 2**). Specifically, we found that when mean nestling mass at our field site was higher, the effect of coloration on nestling mass was more strongly positive **(Figure 5**, Pearson r = 0.90, df=4, p=0.02) However, males that were experimentally challenged did not respond differently based on their coloration (**Figure 6**, $\beta_{color*Treatment}$= 0.001 (p=0.996), $F_{2,19}$=3.97, p=0.02, R^2=0.29), even though our treatment reduced nestling mass (Additive model: β_{color}= -0.37 (p=0.04), $\beta_{treatment}$=-1.90 (p<0.01),

$F_{2,19}$=6.28, p=<0.01, R^2=0.33). However, the effect of coloration on nestling mass in our experimental treatment was consistent with our broad patterns of the honesty of blue coloration across years and seasons (Pearson r (with experimental males) = 0.86, df=5, p=0.02).

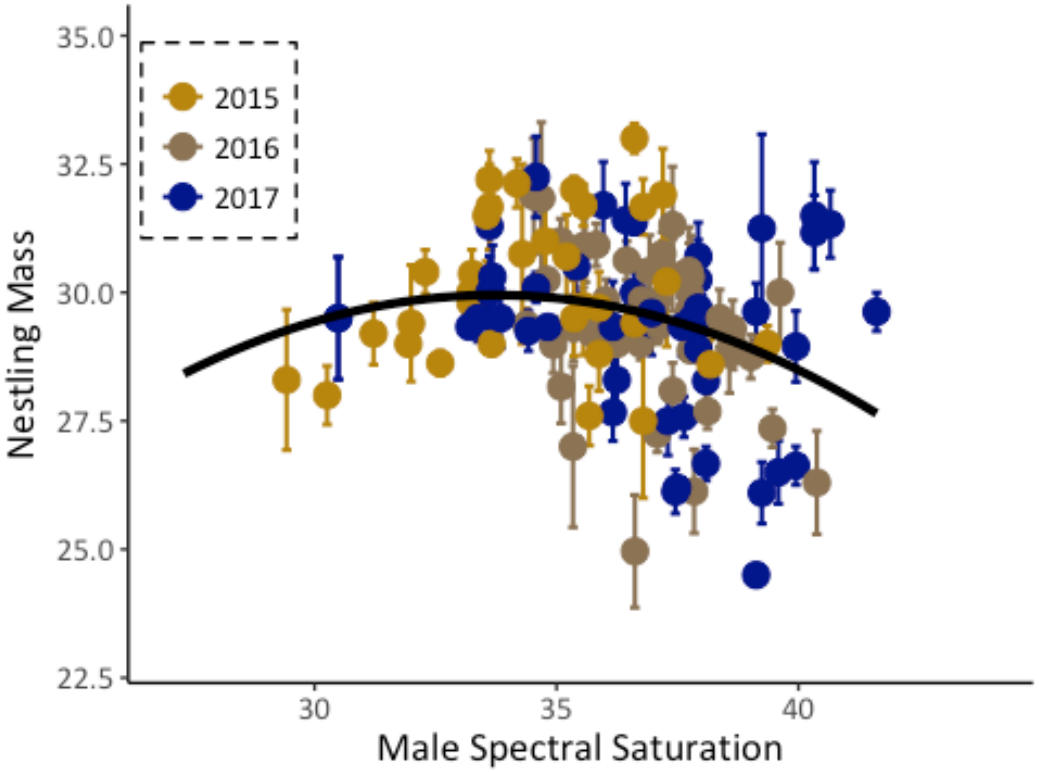

Figure 3 The overall relationship between male coloration and the average mass of nestlings at his nest. Line represents the results of a mixed effects model including a random effect of male ID.

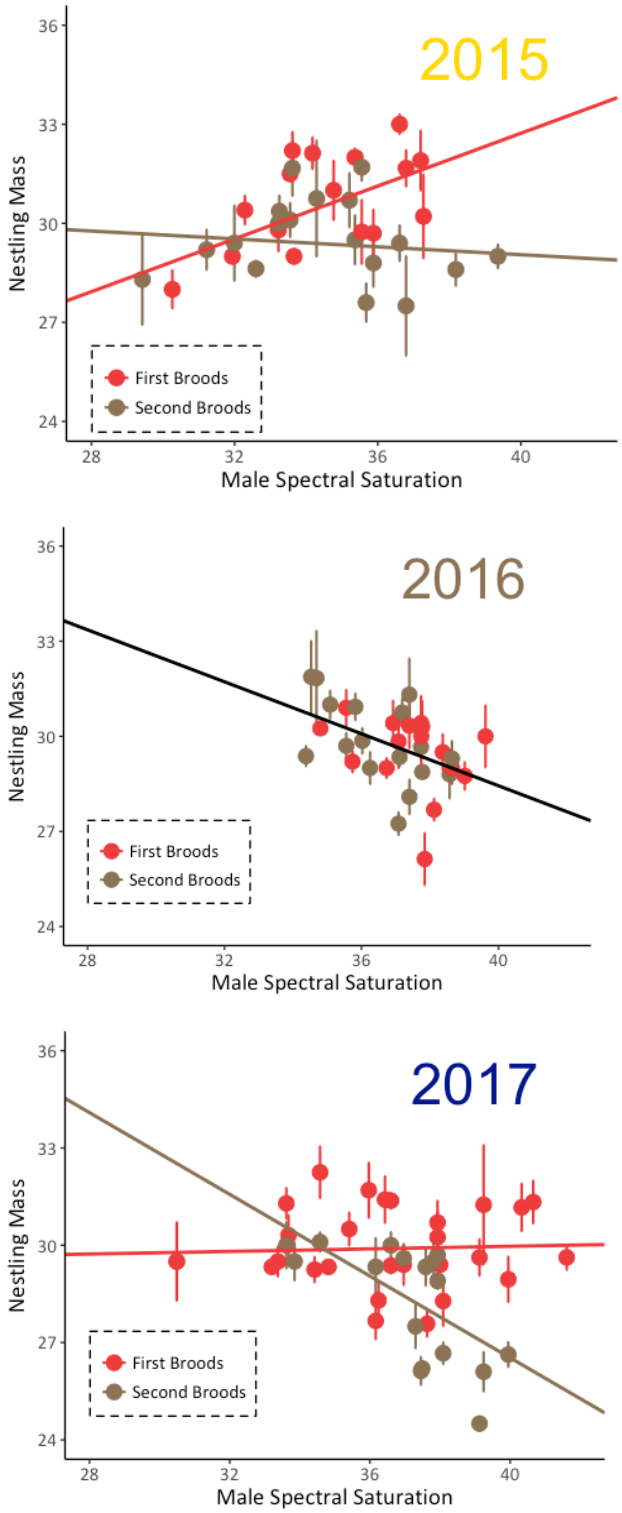

Figure 4 Variation in the relationship between male spectral saturation and nestling mass across years and seasonal timing.

Year	Brood #	β ± SE	Model F_{df}, (p)
2015	1	0.39 ± 0.15	$6.31_{1,14}$, (0.02)
2015	2	-0.06 ± 0.12	$0.22_{1,16}$, (0.61)
2016	1	-0.28 ± 0.24	$1.88_{1,14}$, (0.18)
2016	2	-0.49 ± 0.21	$5.60_{1,15}$, (0.03)
2017	1	0.02 ± 0.08	$0.06_{1,26}$, (0.80)
2017	2	-0.67 ± 0.20	$11.41_{1,15}$, (<0.01)

Table 2 Estimated effect sizes for male coloration on nestling mass across years and seasons (first vs. second brood).

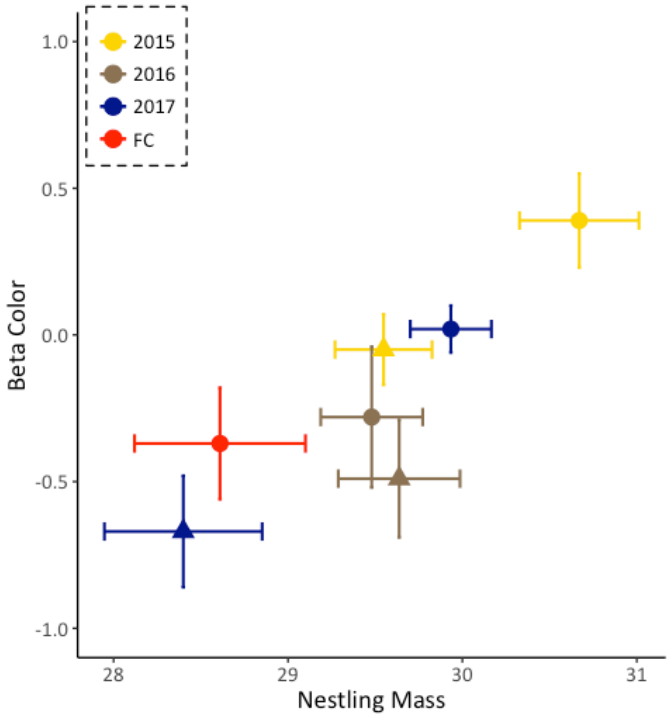

Figure 5 The relationship between the effect size for male coloration and nestling mass across years and seasons. Error bars represent standard error. First broods are denoted by circles, second broods are triangles.

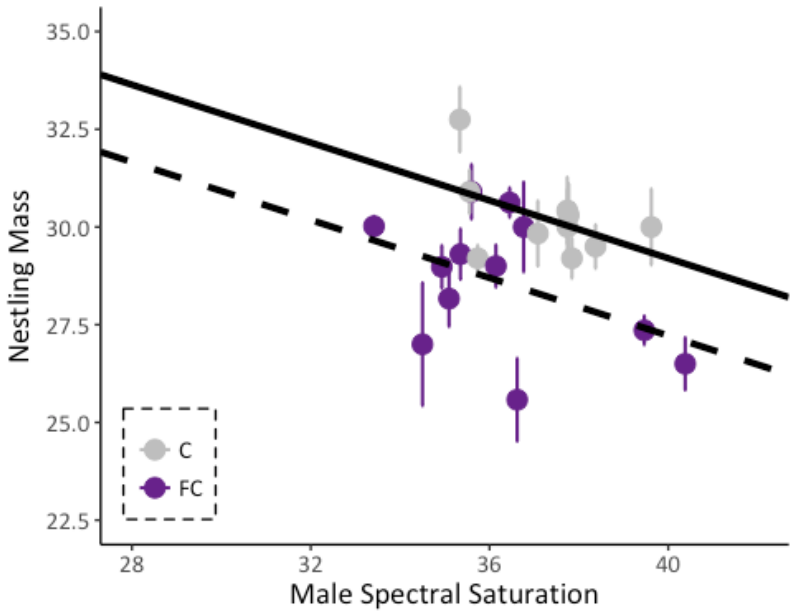

Figure 6 The effect of male coloration on nestling mass during experimental feather clip challenge (2016).

Discussion

The ability of blue coloration to predict direct benefits varied with reproductive investment across years and seasons. When reproductive investment was high, male mountain bluebirds with more saturated colors invested more in reproduction and produced heavier nestlings. However, when reproductive investment across our study site was low, this trend reversed and more colorful males produced lighter nestlings.

We found that variation in reproductive investment covaried with seasonal variation that impacted the distribution of male traits across our study site. When spring progressed quickly and there was less variance in the date of territory establishment, there was also less variance in the coloration of males who obtained territories. Signaling theory predicts that when variance in male traits is high, females should receive relatively more benefits from mating with higher quality males (Kodric-Brown and Brown 1984). Our data partially support this hypothesis in that when mean male trait quality was low in early 2015, the association between male trait quality and offspring mass was strongly positive. However, variance in blue coloration was highest in 2017, and we did not observe the same

positive relationship between male color and nestling mass. This suggests that in years where less colorful males are able to obtain territories, females gain direct benefits from mating with relatively more ornamented males. However, in other years of our study the most elaborately colored males produced the lightest offspring. This warrants further exploration, as it appears that sometimes females pay costs for social pairings with elaborate males.

While we found a strong relationship between reproductive investment and direct benefits, males did not respond differently to our experimental challenge based on their coloration. Variation in responsiveness to challenge has been shown to impact other aspects of sexual selection, though, and should not be ruled out as a source of variation in this or other mating systems (Hill 2011). For example, male soay sheep that invest heavily in horn length in poor environments suffer reduced correlations between horn length and reproductive success as adults (Robinson et al. 2008). This indicates that allocation differences during early development can impact the reproductive benefits associated with sexually selected traits into adulthood. Our data have not ruled out these types of effects. Given that our experimental manipulation predictably altered the relationship between male coloration and direct benefits, it is likely that our experimental perturbation was not strong enough to observe an interaction between male coloration and treatment.

Across years, male mountain bluebirds with intermediate levels of coloration were the most likely to provide direct benefits in the form of offspring quality. Previous work on sexual selection has also documented stabilizing effects of male trait quality on fitness and performance (Gray and Cade 1999, Hunt et al. 2005, O'Brien et al. 2017). These effects could be due to constraint, where males cannot simultaneously optimize both components of fitness, or adaptation, where males adjust their performance based on context (Antonovics and van Tienderen 1991). While our data support adjustments to direct benefits based on context, we cannot completely eliminate temporal constraints regulating trait development. In two out of three of the years of our study, the relationship between male coloration and offspring quality became more negative late in the season. Notably, in late 2017, when nestlings were the lightest, the relationship between male coloration and nestling mass was more negative than in late 2016 or 2015. This suggests adaptive adjustment rather than fixed constraints on resource allocation. However, these effects

may also be due to males partitioning resources between the current reproductive attempt and the onset of seasonal molt, when this signal must be developed to ensure reproductive success in the following year. Overlap between molt and breeding is energetically costly, and birds may be unable to maximize both offspring quality and feather coloration late in the season (Dawson et al. 2000; Foster 1974, 1975; Siikamaki et al. 1994). This temporal constraint is likely strong, and therefore we do not have data to conclusively determine that the changes in nestling mass across our study were adaptive adjustment to reproductive effort.

Overall, future work should focus on manipulating the major contributors to variation in the honesty of sexually selected traits to make robust predictions about when and where we expect traits to be honest indicators of male performance. Here, we identify two potential factors: the distribution of territorial males and changes in optimal reproductive investment. Identifying the causal agents that drive variance in the honesty of sexually selected traits is a crucial goal for future sexual selection research.

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