RH: Indigo Bunting coloration

Condition-dependence of blue plumage coverage in indigo buntings *Passerina cyanea*

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Abstract

Bright plumage coloration is seemingly favored by females of avian species with regards to sexual selection. This particular secondary sexual characteristic has been previously shown to be a condition-dependent signal of individual quality among passerines with pigment-based coloration (*i.e*. yellows and reds). In contrast, relationships between structural plumage coloration (*i.e*. blues) and aspects of both physical quality and physiological function have been understudied. Using free-living Indigo Buntings (*Passerina cyanea*) as a study species, we compared the percentage of blue feather coverage to innate immune responses, antioxidant capacity, stress physiology, reproductive physiology, and parasitism. We found the overall percentage of blue feathers on individual birds to be positively correlated with testosterone levels and body condition, while negatively correlated with heterophil to lymphocyte ratio. Birds with more blue coloration were also less likely to harbor blood parasites. Our results indicate male Indigo Buntings with greater blue plumage coverage have better overall body condition, lower stress, increased testosterone levels, and decreased parasitic susceptibility.

Keywords: coloration; sexual selection; individual quality; ecophysiology; ornithology

1. Introduction

Ornamental colors expressed in sexually dimorphic species are suggested to have evolved in response to sexual selection, whereby color variation functions as a signal influencing mate choice and competition (Darwin, 1871; Andersson, 1994). The handicap principle predicts sexual signals to be reliable indicators of phenotypic or genetic quality, contingent upon the signals being costly to produce and/or maintain (Zahavi, 1975; Andersson, 1982; Nur and Hasson, 1984; Grafen, 1990; Iwasa *et al.*, 1991). Therefore, color signaling is expected to be condition-dependent when high-quality individuals are more capable of affording the costs of elaborate secondary sexual traits (Hamilton and Zuk, 1982, Johnstone, 1995). When individuals choose high-quality mates on the basis of color signals, they can be provided 'direct' (*i.e.* territory access and resources) or 'indirect' (*i.e.* good genes) benefits (Andersson, 1994). Across avian species, there is considerable evidence supporting the female preference for elaborate plumage coloration, coupled with the provision of such benefits (Hill and McGraw, 2006b). Since color expression has been found to be costly in birds (Hill and Montgomerie, 1994; McGraw *et al.*, 2002), ornamental plumage coloration can serve as a condition-dependent signal for female mate choice (Kodric-Brown and Brown, 1984; Fitzpatrick, 1998; Hill, 2006; McGraw, 2008).

Condition-dependence in ornamental plumage colors often relies on whether the colors are derived from feather nanostructure (*i.e.* greens, blues and violets; Shawkey *et al.*, 2003) or pigments such as carotenoids and melanins (*i.e.* yellows, reds, blacks, and browns; Hill and McGraw, 2006a). Carotenoid-based coloration is widely considered costly because pigment deposition depends on dietary access to carotenoids (Brush, 1981; Hudon, 1994; Møller *et al.*, 2000). Similarly, melanin-based color expression can be constrained by limited availability of amino acid precursors and dietary mineral content (Jawor and Breitwisch, 2003; McGraw, 2007). The costs of producing structurally-based colors are less obvious, but formation of feather nanostructure depends on balances between protein synthesis and breakdown of keratin (Jovani and Blas, 2004; Hill, 2006). Indeed, both pigment and structural color expression can conditionally signal foraging ability and/or resistance to nutritional stressors as determined by measures of body condition (Keyser and Hill, 1999; Doucet, 2002; McGraw *et al.*, 2002; Griggio *et al.*, 2009).

Aspects of physiological condition can also be assessed in ornamental plumage coloration; such that female mate choice is influenced by males advertising self-maintenance capacities. Variation in carotenoid-based coloration can reflect levels of oxidative stress and immune function, since limited stores of carotenoid pigments also function as antioxidants and immunostimulants (Lozano, 1994; Chew, 1996; Olson and Owens, 1998, Blount *et al.*, 2003). Alternative mechanisms link melanin pigment deposition to antioxidant and immune function, suggesting physiological condition may also be involved in melanin-based coloration (Moreno, 2005). In contrast, research addressing physiological aspects of self-maintenance in structurally-based coloration has been relatively neglected, with only one study showing color expression to be related to immunocompetence (Griggio *et al.*, 2010).

Further, parasitic infection is thought to be reflected by ornamental plumage coloration as females likely prefer to mate with parasite-free males, which may in turn provide direct benefits (*i.e.* avoidance of parasite transmission; Clayton, 1991) and/or indirect benefits (*i.e.* parasite resistance genes; Hamilton and Zuk, 1982). Under parasite-mediated sexual selection, several cases of condition-dependence for parasite susceptibility have been demonstrated in pigment-based signaling systems (Møller *et al.*, 1999; Mougeot, 2010). The potential for structural coloration to be mediated by such sexual selective pressures has received little attention and current studies report conflicting relationships across species (Doucet and Montgomerie 2003; Zirpoli *et al.*, 2013).

Reproductive physiology may also contribute to condition-dependent signaling since sex hormones have been implicated as developmental mechanisms involved in the organization and expression of sexually dimorphic traits, such as plumage coloration during molting (Kimball, 2006). Under the handicap principle, the immunocompetence handicap hypothesis predicts the expression of secondary sexual traits in males to be mediated by testosterone levels, which can down-regulate immune function (Folstad and Karter, 1992). Here, only individuals with high-quality immune systems may be capable of affording elevated testosterone levels for the development of enhanced ornamental plumages. However, other physiological conditions (*i.e.* body condition, parasite infection) may influence the trade-offs between testosterone production, self-maintenance, and sexual trait expression (Pérez-Rodríguez; 2006). Thus, if testosterone is immunosuppressive, lower body condition may indicate lower levels of testosterone and decreased expression of plumage coloration. Few studies have explored the potential for tradeoffs between physiological condition, testosterone levels, and ornamental plumage coloration, as many correlational approaches focus only on relationships between testosterone and individual variation in sexual signals (Owens and Short, 1995; Kimball, 2006; Peters, 2007; Bókony *et al.*, 2008). Further, whether or not testosterone and other physiological conditions are involved in the expression of structural plumage ornaments in male birds remains unclear (Roberts *et al.,* 2009; Siefferman *et al.,* 2013).

Altogether, the condition-dependent relationships mentioned support the possibility of structural colors signaling various aspects of mate quality, but most studies neglect holistic analyses of physiology and overlook other biologically relevant aspects of color signals. In this study, we examined potential correlations between the percentage of blue plumage coverage (percent blue) in male Indigo Buntings (*Passerina cyanea*) and aspects of individual quality including: (1) body condition, (2) immunocompetence, (3) stress physiology, (4) reproductive physiology, and (5) blood parasite susceptibility. We tested the hypothesis that percent blue in male Indigo Buntings is an indicator of overall individual physical, physiological, and immune quality and could thus serve as a condition-dependent signal for sexual selection.

2. Methods

2.1 Study Species

The Indigo Bunting is a small, socially monogamous songbird that tends to reside in brushy forest edges, open deciduous woods, second growth woodland, and farmland habitats (Sibley and Burt, 1991). As a sexually dimorphic species, Indigo Buntings undergo a series of molts throughout the year. During the breeding season (May-August), the adult male plumage appears as a range of vivid blues. Specifically, the wings and tail are black with cerulean blue edges, whereas the head is indigo. During the nonbreeding seasons (October-March), adult males appear mostly brown while adult females are brown year-round. Immature birds typically resemble females in coloring, but a juvenile male may have hints of blue on the tail and shoulders as well as darker streaks on the underside.

2.2 Field Capture Measurements and Samples

We employed mist nets to capture and band adult male Indigo Buntings (n = 40) from four different conservation sites in Macon County, IL, U.S.A., between April 2013 and October

2014. Upon capture, we qualitatively determined sex, age, presence of ectoparasites, symptoms of disease, and reproductive status. We also quantified physical structures, mass, fat storage, and percent blue of the plumage coloration. To obtain plasma and assess physiological condition, we extracted blood samples (~140µl) via venipuncture of the brachial vein.

Given the evidence for age-related effects on plumage coloration (Siefferman *et al.*, 2005; Budden and Dickinson, 2009; Edler and Friedl, 2012; Nam *et al.,* 2016), we only sampled breeding adult males that had molted into their nuptial plumage. This allowed us to assess the overall percentage of blue plumage that is potentially relevant to intraspecific interactions and sexual selection overall. For this assessment, we quantified percent blue as a visual approximation of the overall blueness of each individual based on a (0-100) scale. T. Wilcoxen was present for the capture and assessment of percent blue for all birds in this study. In addition, no indicators of percent blue were included in the identification of the blood samples in the lab; therefore, the physiological values were determined blind to the coloration.

2.3 Blood Analysis

For innate immune measures, we conducted an *Escherichia coli* bacterial killing assay (BKA) on blood plasma to determine levels of resistance to infection. We completed this *in vitro* challenge by inoculating a 10µl plasma sample in minimum essential media with an *E. coli* solution (10µl at approximately 250 CFU) and completing a challenge for 20 min at 41 C. We added the mixture to tryptic soy agar and placed each sample under incubation for 24 h at 37 C to promote *E. coli* growth. We determined killing ability by measuring the difference in percentage of bacteria grown in each sample compared to that of a control without plasma (10µl *E. coli* solution in 110µl minimum essential media; Wilcoxen *et al.*, 2010).

For measures of stress physiology, we used a total antioxidant capacity assay (TAC;

OxiSelect TAC, CellBioLabs, Inc., San Diego, CA), which provided us with an indication of the overall capability to counteract reactive oxygen species, resist oxidative damage, and combat oxidative stress. This *in vitro* test is based on the reduction of copper (II) to copper (I) by antioxidants in plasma samples. Upon reduction, the copper (I) ion further reacts with a coupling chromogenic reagent that produces a color with a maximum absorbance at 490 nm. We compared the net absorbance values of the antioxidants with a standard curve of known uric acid concentrations to determine the TAC for each bird. We additionally examined blood smears stained with Wright-Giemsa to determine heterophil to lymphocyte ratios (H:L; Davis *et al.*, 2008) for each bird. Under chronic stressors, an elevation in glucocorticoid hormones, such as corticosterone (CORT; the primary avian stress hormone) can impair the presentation of MHC molecules that are necessary for the generation of lymphocyte responses, including lymphocyte proliferation (Zwilling *et al.*, 1990; Truckenmiller *et al.*, 2005). As such, a lower (H:L) ratio would indicate less stressful conditions that potentially permit greater immunocompetence (Davis *et al.*, 2008).

To assess reproductive physiology, we employed an enzyme-linked immunosorbent assay (ELISA; Arbor Assays, Ann Arbor, MI), which measured circulating testosterone levels from 20µl of plasma. This ELISA is based on competitive binding between testosterone antibodies and testosterone from the plasma samples. We also assessed individual susceptibility to parasitic infection by analyzing blood smear slides to confirm the presence or absence of endoparasites belonging to four major parasitic genera: *Plasmodium*, *Haemoproteus*, *Leukocytozooan*, and *Microfilaridae.* To determine if any parasites among those four genera were present, we started at the top of the widest point of the blood smear and scanned to the bottom of the slide without moving the field of view from side to side. We recorded a “positive” for any of the four parasites if at least one of the related species was present.

2.4 Statistical Treatment of Data

By reducing the highly correlated morphometric variables into a single factor using principal component analysis, we established a body condition index (BCI; Wilcoxen *et al.*, 2010). This extracted principal component included the tail, wing, and tarsus (lower leg length) and measurements regressed on mass, with the residuals serving as the body condition index (Eigenvalue = 82.4%). To assess relationships between percent blue and the physiology and condition metrics, we ran multiple regression analyses for the dependent variable, percent blue, with BKA, TAC, H:L, BCI, and testosterone levels as the independent variables. We also used a binary logistic regression analysis to determine if percent blue was an indicator of parasitism. For this test, the categorical variable "parasitized: yes or no" was the dependent variable and percent blue was incorporated as the continuous independent variable. We completed all analyses using SPSS 21.0 (IBM Corp., 2012).

3. Results

We found a positive correlation between body condition and the percent blue component of the Indigo Bunting feathers ($r = 0.486$, $n = 40$, P = 0.026; Fig. 1), with approximately 23.6% of the variation in percent blue explained by body condition ($r^2 = 0.236$). We found a significant negative linear relationship between percent blue and the heterophil to lymphocyte ratio ($r = -0.316$, n = 40, P = 0.041; Fig. 2), with 10.0% of the variation in percent blue explained by heterophil to lymphocyte ratios ($r^2 = 0.100$). We found a significant positive linear relationship between percent blue and circulating testosterone levels ($r = 0.611$, n = 40, P = 0.006; Fig. 3), with approximately 37.4% of the variation in percent blue explained by testosterone ($r^2 = 0.374$).

We found no relationships between *E. coli* killing ability (BKA) and percent blue scores (r = -0.13, r^2 = 0.018, n = 40, P = 0.232), nor did we find a relationship between total antioxidant capacity and percent blue scores (r = 0.05, r^2 = 0.003, n = 40, P = 0.823).

We found percent blue to be a significant predictor of parasitism (Fig. 4). For every 11.1% (B = -0.111) reduction in blueness, males are approximately 1.9 (Exp(B) = 1.89) times more likely to be parasitized, with approximately 22.7% of the variation in parasite presence explained by variation in percent blue (Cox and Snell, r^2 = 0.227, P = 0.011).

4. Discussion

Measurements of blue plumage coverage among male Indigo Buntings reveal extensive individual variation in ornamental coloration, suggesting the potential for sexual selection on this trait. Further, percent blue estimates are strongly correlated with important physiological metrics (BCI, H:L, testosterone, and parasitism), but not related to innate immunity or antioxidant capacity. Taken together, male Indigo Buntings with greater blue plumage coverage have better overall body condition, lower stress, increased testosterone levels, and are less likely to be infected with blood parasites. Our hypothesis that blue plumage coverage in male Indigo Buntings is a condition-dependent signal for sexual selection was thus supported.

Our blue plumage coverage results support previous studies suggesting structural plumage colors can be indicative of individual body condition. Since blue plumage expression depends on the body condition of a male during molting (Keyser and Hill, 1999), conspecifics assessing male ornamentation can receive information about a male's past nutritional history. This appears to be the case in Blue Grosbeaks (*Passerina caerulea*) as increased area of blue plumage is correlated with larger body size, larger territories, and greater prey abundance (Keyser and Hill, 2000). Other components of structural plumage coloration are also predictors of body condition in nestling Blue Tits (*Cyanistes caeruleus*; Johnsen, 2003), Steller's Jays (*Cyanocitta stelleri*; Siefferman *et al.*, 2013), Blue-black Grassquits, (*Volatinia jacarina*; Doucet, 2002) and Satin Bowerbirds (*Ptilonorhynchus violaceus*; Doucet and Montgomerie, 2003). As such, the relationships demonstrated in our study and others suggest structural colors may function as signals of nutritional condition, foraging ability, and the environment in which a male was raised.

Given our findings that percent blue is a significant predictor of parasite susceptibility, we add support to the growing body of literature suggesting structural plumage coloration may serve as a condition-dependent signal mediated by parasite-mediated sexual selection. Of the few cases demonstrating structural color signaling for parasite susceptibility, there remains only species-specific relationships. The intensity of infection from blood parasites is similarly predicted by structural plumage coloration in Satin Bowerbirds (*Ptilonorhynchus violaceus*; Doucet and Montgomerie, 2003). However, parasite load is positively related to structural plumage expression among Steller's Jays (*Cyanocitta stelleri*; Zirpoli *et al.*, 2013). When considering the immune system, male Indigo Buntings with a greater percent blue exhibited lower H:L ratios as expected in relation to the relative likelihood of being parasitized (Davis *et al.*, 2004; Lobato *et al.*, 2005). An individual with a greater blue plumage may thus be undergoing less physiological stressors, permitting more effective immune responses to other pathogenic infections, including future parasitic encounters (Davis *et al.*, 2008). However, relationships between plumage coloration, stress levels, and immunity are far reaching considering blue plumage coverage, antioxidant capacity, and bacterial killing ability were not correlated.

Physiological relationships predicted by the immunocompetence handicap hypothesis are partially supported by our findings since percent blue was positively correlated to testosterone levels, but not related to innate immunity. Higher circulating testosterone levels could still serve as a developmental mechanism involved in the expression of sexual traits, given other aspects of physiological condition (Pérez-Rodríguez, 2006). Variation in body condition and parasite infection may explain variation in testosterone production and expression of blue plumage coverage. Individuals with greater blue plumage coverage, healthy body condition, and low likelihood of parasitic infection could be affording the costs of immunosuppression. Conversely, individuals in poor body condition that are susceptible to parasitic infection could have lower levels of testosterone to minimize the risks of infection and costs of color expression. In Blue Tits (*Cyanistes caeruleus*), testosterone enhanced both immune function and structural coloration contingent upon the condition of the individual (Roberts *et al.*, 2009). However, the development and maintenance of structural color expression does not seem to be affected by testosterone and body condition in Eastern Bluebirds (*Sialia sialis*; Grindstaff *et al.*, 2012; Siefferman *et al.*, 2013). If blue plumage coverage functions as a testosterone-mediated signal, it could indicate variable aspects of current physiological state (Pérez-Rodríguez *et al.*, 2006), but the causal relationship remains to be resolved across species. Alternatively, male Indigo Buntings with more blue plumage overall may be subject to more frequent interactions with females and reproductive activity, eliciting an increase in testosterone production. Therefore, the correlation between testosterone and percent blue may implicate condition-dependence or it may be a product of increased reproductive activity.

This study reveals blue plumage coverage to be a reliable indicator of many physical and physiological measures in male Indigo Buntings. Our results are in line with previous studies that found condition-dependent relationships between aspects of structural plumage coloration and individual quality, suggesting blue plumage coloration in this species may be a sexually selected signal. Multiple aspects of structural plumage ornamentation in this species may serve as condition-dependent signals that can be assessed by conspecifics during mate choice or competition. To complement our findings, we suggest the pursuit of mate choice studies to confirm that females of this species select mates on the basis of various measures of structural plumage coloration. Genetic paternity analyses could further support for sexual selection theory by testing the prediction that more highly ornamented males experience greater reproductive success. Considering 20-40% of Indigo Bunting offspring are borne out of extra-pair copulations (Payne, 1992), extra-pair paternity must also be accounted for if the benefits of structural color signals are to be accurately assessed in terms of overall fitness.

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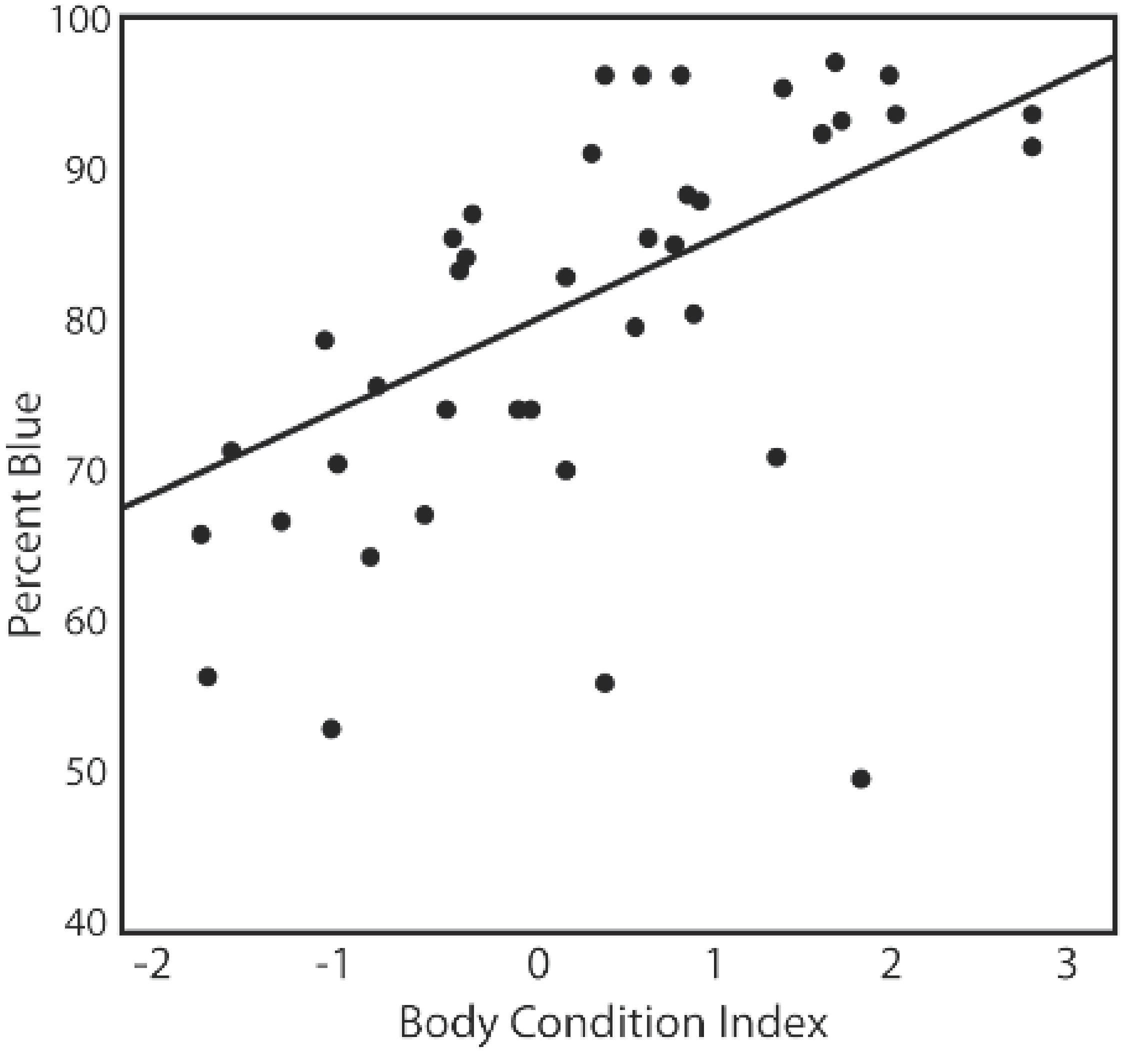
Figure Legends

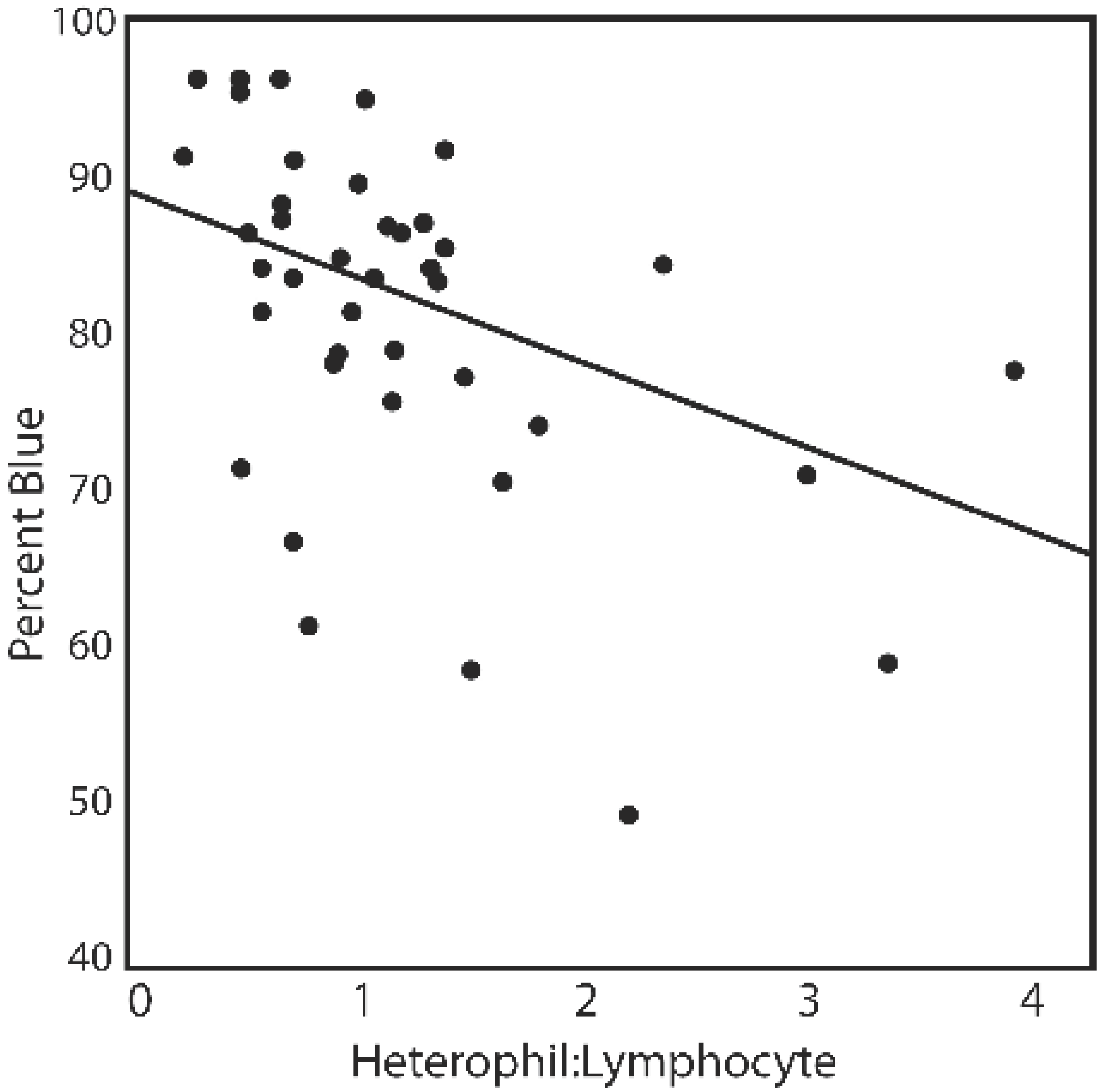
Figure 1.— Linear regression model of percent blue of Indigo Bunting (*Passerina cyanea*) plumage coloration with respect to body condition index

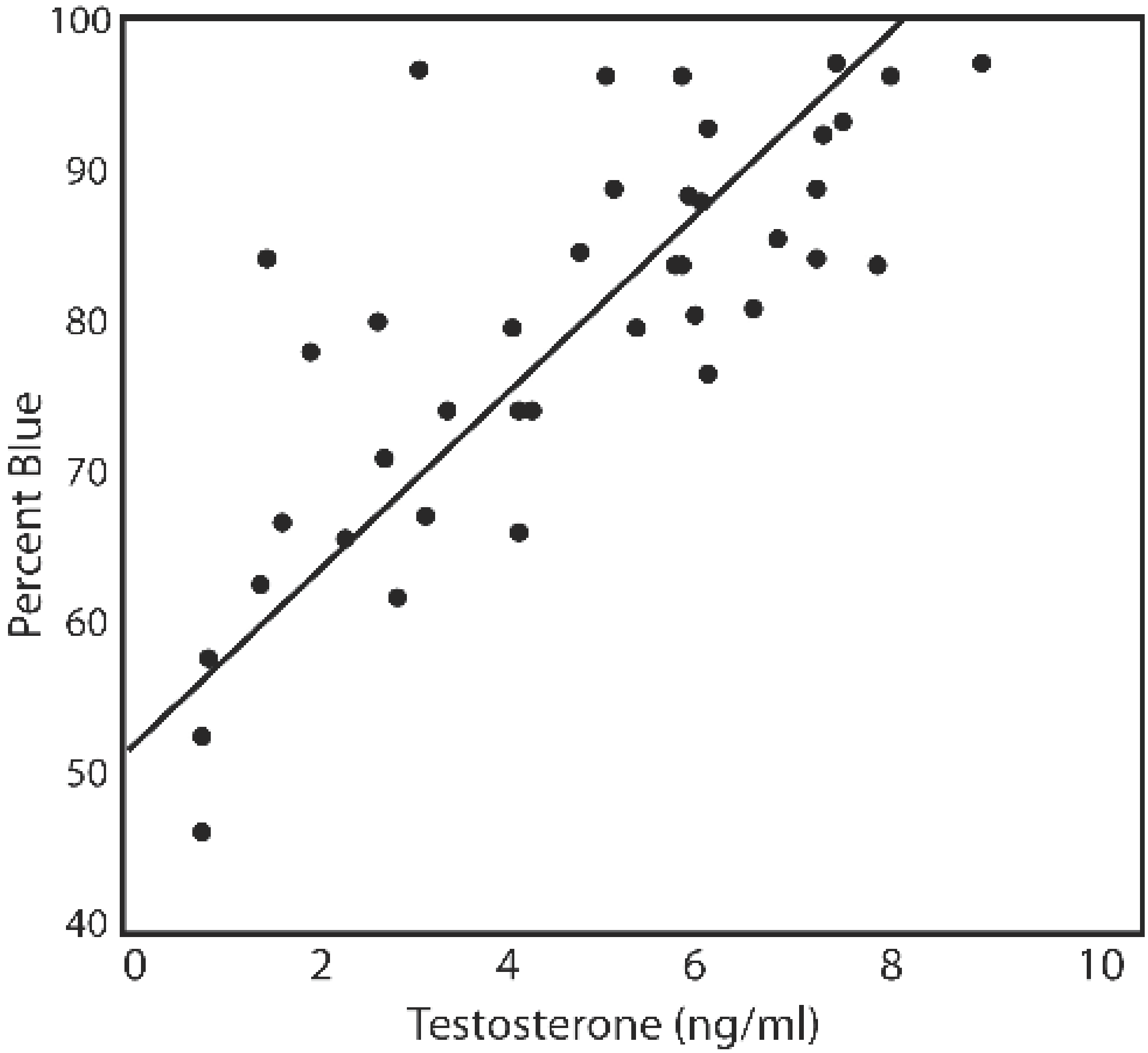
Figure 2.— Linear regression model of percent blue of Indigo Bunting (*Passerina cyanea*) plumage coloration with respect to heterophil to lymphocyte ratio.

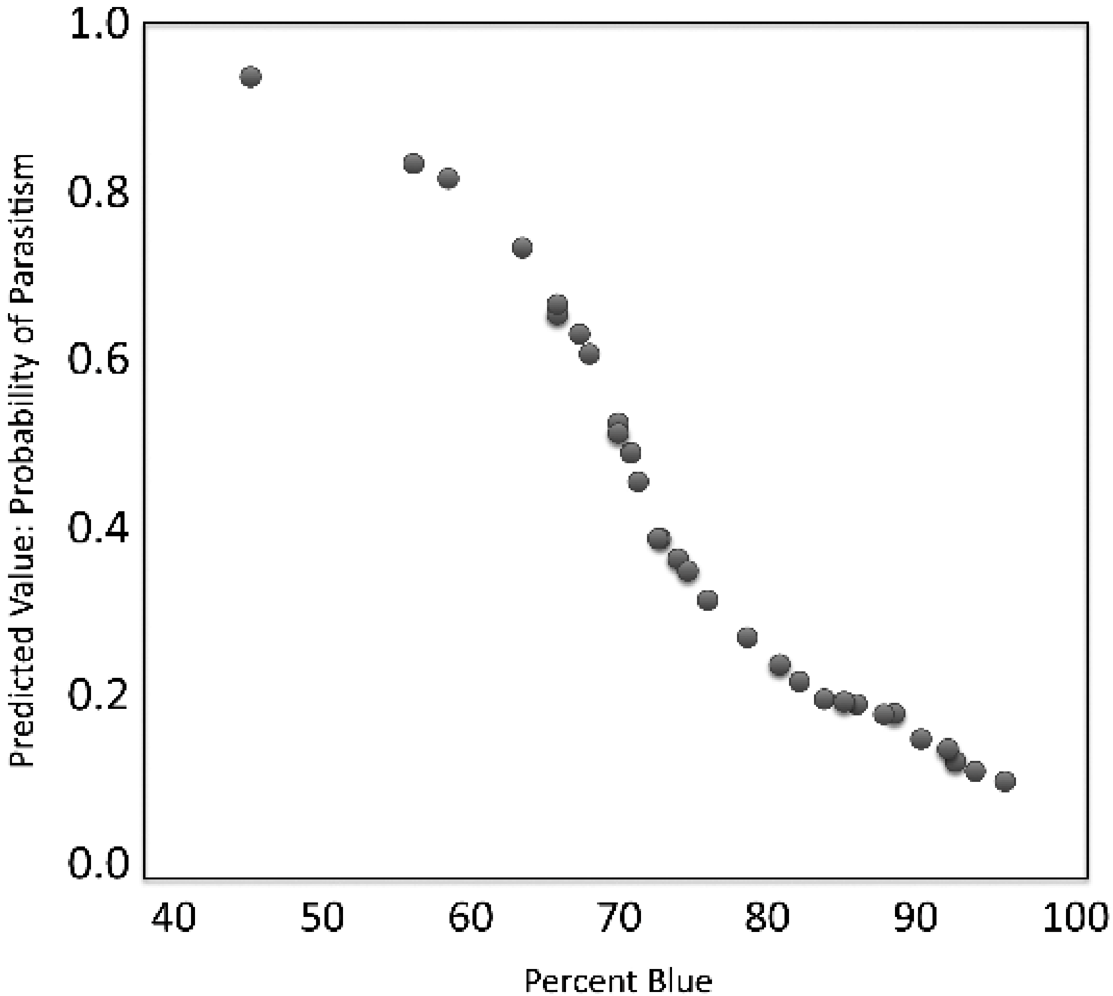
Figure 3.— Linear regression model of percent blue of Indigo Bunting (*Passerina cyanea*) plumage coloration with respect to testosterone (ng/ml).

Figure 4.— Binary regression analysis of parasitism probability with respect to percent blue of Indigo Bunting (*Passerina cyanea*) plumage coloration


Percent Blue
Body Condition Index
100
90
80
70
60
50
40
-2
-1
0
1
2
3


100
90
80
70
60
50
40
0
1
2
3
4
Percent Blue
Heterophil:Lymphocyte


100
90
80
70
60
50
40
Percent Blue
0
2
4
6
8
10
Testosterone (ng/ml)


1.0
0.8
0.6
0.4
0.2
0.0
40
50
60
70
80
90
100
Percent Blue
Predicted Value: Probability of Parasitism