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Relationships among plumage coloration, blood selenium concentrations, and immune responses of adult and nestling tree swallows

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Abstract

In a number of taxa, males and females both display ornaments that may be associated with individual quality and could be reliable signals to potential mates or rivals. We examined the iridescent blue/green back and white breast of adult tree swallows (*Tachycineta bicolor*) to determine if plumage reflectance was related to adult or offspring immune responses. We simultaneously addressed the influence of blood selenium levels and the interaction between blood selenium and plumage coloration on adult and nestling immunity. Selenium is a well-known antioxidant necessary for mounting a robust immune response but its importance in wild birds remains poorly understood. In females, the brightness of white breast coloration was positively associated with bactericidal capacity, but there was no association with blood selenium. In contrast, male bactericidal capacity was associated with an interactive effect between dorsal plumage coloration and blood selenium concentrations. Males with bluer hues and greater blue chroma showed increased bactericidal capacity as blood selenium concentrations increased, while bactericidal capacity declined in greener males at higher blood selenium concentrations. In nestlings, bactericidal capacity was positively associated with nestling blood selenium concentrations and white brightness of both social parents. These results suggest that white plumage reflectance is indicative of quality in tree swallows and that greater attention should be paid to the reflectance of large white plumage patches. Additionally, the role of micronutrients, such as selenium, in mediating relationships between physiology and signals of quality, should be explored further.

Summary Statement

Selenium and structural plumage coloration are related to innate immunity in adult and nestling tree swallows.

Keywords: bactericidal capacity, micronutrient, plumage coloration, selenium, tree swallow

Introduction

In many animals, individuals display elaborate ornaments that are used as signals to conspecifics (Andersson, 1994). Because these ornaments are often costly to produce or maintain, only individuals in the best condition will be capable of possessing the most elaborate ornaments, making ornament expression a reliable indicator of phenotypic and/or genetic quality (Zahavi, 1975; Hamilton and Zuk, 1982; Grafen, 1990). In males, these ornaments appear to be maintained primarily by sexual selection because more ornamented males are preferred mates or are superior competitors, and as a result obtain more mating opportunities and greater reproductive success (Andersson, 1994). However, in a number of taxa females display ornamentation that is also associated with individual quality (Amundsen and Pärn, 2006; Kraaijeveld et al., 2007). Traditional sexual selection likely plays a limited role in maintaining female ornamentation because female reproductive success is largely limited by their ability to produce young rather than their access to mates (LeBas, 2006; Tobias et al., 2012). Female ornamentation is more likely maintained by social selection, competition among conspecific females for resources other than mates (LeBas, 2006; Tobias et al., 2012). Because different selective pressures maintain ornamentation in each sex, it is possible that similar ornaments are related to different aspects of individual and/or offspring quality in each sex (Massaro et al., 2003; Kekäläinen et al., 2010; Kelly et al., 2012; Pickett et al., 2013).

In many birds, both sexes display colorful plumage that may be produced by pigments deposited in feathers (carotenoid and melanin based colors) or by the feather microstructure (structural coloration). One aspect of individual quality that plumage coloration is frequently linked to is the ability to resist parasite infection or mount an

immune response (Dunn et al., 2010; Kelly et al., 2012). While previous work focused largely on pigment-based, particularly carotenoid coloration, recent work has shown that structural coloration may also be related to immunity or levels of parasitism (Doucet and Montgomerie, 2003; Bonato et al., 2009; Griggio et al., 2010). For example, in male satin bowerbirds (*Ptilonorhynchus violaceus*) , brighter blue or greater UV chroma is associated with lower parasite loads (Doucet and Montgomerie, 2003). Male ostriches (*Struthio camelus*) and south polar skuas (*Catharacta maccormicki*) with larger or more colorful white plumage patches mount a stronger humoral immune response (Bonato et al., 2009; Hanssen et al., 2009). Finally, stronger cutaneous immune responses (response to phytohaemagglutinin (PHA) injection) have been associated with greater UV reflectance of green plumage in budgerigars (*Melopsittacus undulates*) (Griggio et al., 2010). Only recently have studies examined the relationship between female coloration and immunity and have primarily focused on white plumage patches. In female common eiders (*Somateria mollissima*), smaller white wing bands were associated with immunosuppression (Hanssen et al., 2006) while larger wing patches were associated with lower parasite loads in Eurasian black-billed magpies(*Pica pica*) (Blanco and Fargallo, 2013). A greater number of white spots and spots with greater UV reflectance were associated with a stronger cutaneous immune responses in female Diamond firetails (*Stagonopleura guttata*) (Zanollo et al., 2012). It seems that in both sexes, structural plumage coloration may be associated with "good genes" for greater immunocompetence or more colorful individuals may be in better condition and thus mount stronger immune responses.

The plumage coloration of adults may also be related to immune responses of their offspring. For instance, white plumage coloration of male ostriches was positively

associated with the humoral immune response of their offspring (Bonato et al., 2013). In female great tits (*Parus major*), more immaculate white cheek patches were associated with greater cutaneous immune responses in offspring (Remeš and Matysioková, 2013). Relationships between parental coloration and offspring immunity could arise in several ways. First, more colorful adults may have better immune responses and if the basis for immunity is heritable, then offspring of more colorful parents will also have stronger immune responses (Folstad and Karter, 1992; von Schantz et al., 1999). Transgenerational epigenetic effects can also enhance offspring immunity (Grindstaff et al., 2003; Hasselquist and Nilsson, 2009; Clairardin et al., 2011) and could be associated with parental coloration. Alternatively, more colorful adults may provide greater or higher quality resources to young (Linville et al., 1998; Siefferman and Hill, 2003; Garcia-Navas et al., 2012) which could enhance offspring immunity because mounting an immune response can be energetically costly and influenced by condition (Norris and Evans, 2000; Lifjeld et al., 2002; Palacios et al., 2012; Pickett et al., 2013).

One aspect of nutritional condition that may influence the strength of the immune response is the availability of micronutrients such as carotenoids, vitamins, or trace elements (reviewed in Hasselquist and Nilsson, 2012). These factors may directly enhance the immune response by facilitating immune cell proliferation, production of cytokines, activity of immune cells, or altering gene expression (reviewed in Wintergerst et al., 2007; Hasselquist and Nilsson, 2012). These factors may also influence the strength of the immune response through their antioxidant activity and ability to scavenge reactive oxygen species that can be immunosuppressive (reviewed in Wintergerst et al., 2007; Hasselquist and Nilsson, 2012). In birds, the majority of

micronutrient studies have focused on the role of carotenoids in immunity because these pigments are also used extensively in plumage coloration (Lozano, 1994; von Schantz et al., 1999). While carotenoid supplementation may be beneficial, high carotenoid intake may be detrimental (Huggins et al., 2010; Giraudeau et al., 2013), suggesting that there may be an optimal level of micronutrient supplementation. Given that brighter plumage coloration is associated with greater antioxidant defenses in some species (Perez-Rodriguez et al., 2010; Marko et al., 2011), it is possible the point at which micronutrients become toxic varies among individuals. Additionally, the role of trace elements in modulating the immune response has not been well examined in wild birds, despite the fact that optimal concentrations of some elements, such as selenium, are necessary for mounting robust immune responses and are important components of enzymes with antioxidant functions (Surai, 2002; Surai, 2006; Wintergerst et al., 2007; Huang et al., 2012).

Tree swallows (*Tachycineta bicolor,* Vieillot, 1808) provide a model system for examining the relationships between structural coloration, immunity, and micronutrient status. All male tree swallows have iridescent blue-green dorsal plumage with white ventral plumage (Robertson et al., 2011). Females display delayed plumage maturation; after-second year (ASY) females have blue-green dorsal plumage identical to males while second-year (SY) females have brown dorsal plumage and both have white ventral plumage (Stutchbury and Robertson, 1987b). Because of this dichotomy in female coloration, most studies to date have focused on ASY females and males and found that plumage coloration is a signal used in mate choice and associated with aspects of individual quality. Tree swallows pair assortatively by plumage brightness (Bitton et al., 2008) and males that are brighter and/or older produce more extra-pair

offspring (Bitton et al., 2007). Males that are brighter, with lower wavelength hues are more likely to be recaptured in subsequent breeding seasons, suggesting they have greater site fidelity or higher survival, than males that are duller, with green shifted hues (Bitton and Dawson, 2008). ASY females with bluer plumage hues fledge more offspring while brighter dorsal plumage is positively associated with mean egg mass (Bitton et al. 2008, but see Bentz and Siefferman 2013). However, another study found that more colorful ASY females produced smaller swellings in response to PHA injection, suggesting they have weaker immune responses (Bentz and Siefferman, 2013). Overall, these results suggest tree swallows that are brighter with bluer hues are higher quality than dull (lower brightness) individuals with green shifted hues (but see Bentz and Siefferman 2013). However, no study to date has examined the reflectance of white breast plumage to determine if it is a signal of quality or how parental coloration relates to offspring immunity. Additionally, concentrations of the micronutrient selenium were positively associated with the bactericidal capacity of nestling plasma (Beck et al., 2014b) and with wing growth (Beck et al., 2015) in this species, revealing the need for further examination of the role of selenium in immunity.

The purpose of this study was three-fold. We examined two types of structural plumage coloration, the iridescent blue/green back and white breast, of both sexes of adult tree swallows. We first examined variation in plumage coloration between sexes and determined if the sexes pair assortatively by coloration. Second, we determined if structural plumage coloration related to the immune response of adults or that of their nestlings by examining bactericidal capacity (adults and nestlings) and the response to injection of PHA (nestlings only). In this case, we were specifically interested in whether we would find different relationships between coloration and immunity in the

two sexes given that different selective pressures maintain coloration in males and females (LeBas, 2006; Kelly et al., 2012). Previous studies found individuals with brighter white plumage produced stronger immune responses (Bonato et al., 2009) and that tree swallows with lower wavelength hues and brighter blue plumage are of higher quality (Bitton et al., 2007; Bitton and Dawson, 2008; Bitton et al., 2008). Thus, we predicted that adults with brighter white breast plumage and/or with dorsal plumage that was brighter, with bluer hues and greater blue chroma would have greater bactericidal capacity and produce young with stronger immune responses than adults with dull breast plumage or dorsal plumage with greener hues and greater green chroma. Third, we examined blood concentrations of the micronutrient selenium, to determine if it related to immune function. We examined a range of selenium concentrations by sampling birds at colonies impacted by a spill that increased selenium availability and at nearby reference colonies with background selenium concentrations. We also examined interactive effects between blood selenium concentrations and coloration to address the possibility that more attractive, higher quality individuals or their offspring respond differently to selenium supplementation than less colorful individuals. We predicted that individuals with greater blood selenium concentrations would mount stronger immune responses than those with lower blood selenium concentrations.

Results

Variation in plumage coloration, principal components analysis, and assortative mating

Plumage coloration differed significantly between males and ASY females (Pillai's trace = 0.410, $F_{6, 117}$ = 13.6, P < 0.001). The dorsal plumage of males had lower green chroma, greater blue and uv chroma, and lower hue than that of ASY females (Table 1, all $F_{1, 124} \geq 13.7$, $P < 0.001$). The brightness of blue dorsal plumage did not differ between the sexes nor did the brightness of their white breast plumage (all $F_{1, 124} \leq 0.003$, $P \geq 0.96$). Coloration did not differ significantly between impacted and reference colonies (Pillai's trace = 0.070, $F_{6, 117}$ = 2.8, P = 0.20) nor was there a significant interaction between sex and colony (Pillai's trace = 0.063, $F_{6, 117}$ = 1.3, P = 0.26).

We calculated principal components scores using color data from 603 males and 583 ASY females captured in 2011 and 2012. Principal components analysis produced two principal components that explained 89.3% of the variance in dorsal plumage coloration of adults (Table 2). PC1 explained 65.7% of the variance and received strong positive loadings for green chroma and hue and strong negative loadings for blue and UV chroma. Thus, individuals with high PC1 scores have hues shifted toward higher wavelengths (greener) and greater green chroma while individuals with low PC1 scores have hues shifted toward lower wavelengths (bluer) and greater blue and UV chroma. PC2 explained an additional 23.6% of the variance in blue coloration and received a strong positive loading for blue brightness and a weaker positive loading for blue chroma. Individuals with greater PC2 scores have brighter blue coloration and a greater

proportion of the curve in the blue portion of the spectrum. We used the PC scores to examine assortative pairing in 2012 of 197 pairs of adults. Across the entire study area, males and females paired assortatively by PC1 scores (r = 0.19, P = 0.01) but not by PC2 scores (r = 0.06, P = 0.40) nor by white brightness (r = 0.05, P = 0.50).

Blood selenium concentrations and bactericidal capacity

We quantified the blood selenium concentrations of 29 males, 15 females, and 77 nestlings (one per nest box) and used these three groups (age/sexes) as categories in a two-way ANOVA to determine if they differ in selenium concentrations or bactericidal capacity at exposed (greater selenium bioavailability) and reference (background selenium bioavailability) colonies. We found that blood selenium concentrations were significantly greater at exposed than at reference colonies ($F_{1, 114}$ = 49.2, $P < 0.001$), and differed between adults and nestlings ($F_{2, 114}$ = 93.5, $P < 0.001$), but the interaction between age/sexes and colony was not significant ($F_{2, 114}$ = 2.1, P = 0.13). Tukey post-hoc tests indicated that adults had significantly greater blood selenium concentrations than nestlings (Table 3, all $P \leq 0.001$). Exposed and reference colonies did not differ in bactericidal capacity (Table 3, $F_{1, 114}$ = 0.001, P = 0.98). However, nestlings had significantly lower bactericidal capacity than adults of both sexes ($F_{2, 114}$ = 15.2, $P < 0.001$) regardless of colony (interaction: $F_{2, 126}$ = 2.4, P = 0.10).

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Adult coloration and adult bactericidal capacity

We found that bactericidal capacity of adult plasma was related to different aspects of plumage coloration in female and male tree swallows. For ASY females, the

final model indicated that the brightness of white breast plumage was positively associated with her bactericidal capacity (Table 4, Figure 1, r^2 = 0.31, df = 11, P = 0.05). For males, the final regression model included PC1 scores, blood selenium concentrations, and their interaction (Table 4, r^2 = 0.42, df = 20, P = 0.01). Males with lower PC1 scores (greater blue and UV chroma, hues shifted toward blue) had the greatest bactericidal capacity, but only if they had blood selenium concentrations around 10 μg/g wet mass (wm, Figure 2). In contrast, males with the highest PC1 scores (greater green chroma and hue) with blood selenium concentrations in excess of 10 μg/g wm had the lowest bactericidal capacity (Figure 2). For both sexes, sample collection date, residual body mass, blood selenium concentrations, other aspects of coloration, and other interaction terms were unrelated to bactericidal capacity (Table 4).

Adult coloration and nestling immune responses

We quantified the bactericidal capacity of a single nestling from each of 76 nest boxes, and captured 38 females and 56 males who were attending these boxes. Bactericidal capacity of nestling plasma was related to aspects of plumage coloration in ASY females and social males. For ASY females, the final regression model indicated that Julian sample date, female white brightness, blood selenium concentrations, and the interaction between selenium concentrations and residual body mass were related to nestling bactericidal capacity (Table 5, r^2 = 0.45, df = 31, P = 0.002). We calculated the residuals from a regression of sample date against nestling bactericidal capacity to better focus on the other independent variables. We found that female white brightness had a positive relationship with residual bactericidal capacity (Figure 3A). Additionally, nestlings with low residual body mass and low blood

selenium concentrations had the lowest residual bactericidal capacity while the greatest bactericidal capacity was associated with nestlings with high blood selenium concentrations (Figure 3B). For social males, the final regression model indicated that white brightness and the interaction between white brightness and nestling blood selenium concentrations were related to nestling bactericidal capacity (Table 5, r^2 = 0.30, df = 51, P = 0.004). This result showed that nestlings with greater blood selenium concentrations and a brighter white social male had the greatest bactericidal capacity, whereas young of dull social males and/or with low blood selenium concentrations had low bactericidal capacity (Figure 4). Nestling bactericidal capacity was not related to the bactericidal capacity of the social male (r = 0.06, N = 16, P = 0.81) or female (r = 0.06, N = 12, P = 0.85).

In contrast to bactericidal capacity, the PHA response of nestlings was unrelated to the plumage coloration of the female and social male, selenium concentrations in nestling blood, sample date, residual mass, and the interaction terms (Table 6, final model social male, r^2 = -0.05, df = 17, P = 0.73; final model female, r^2 = 0.05, df = 15, P = 0.38).

Discussion

In a number of avian species as well as many other taxa, both sexes display ornaments that may serve as honest signals of quality to potential mates or rivals (Amundsen and Pärn, 2006; Kraaijeveld et al., 2007). In many cases, these ornaments may be similarly expressed by each sex, but may be the product of different sources of selection, sexual selection in males and social selection in females. Due to these different sources of selection, similar ornaments expressed in both sexes could relate to

different aspects of quality, though only a few recent studies have found such differences (Kelly et al., 2012; Pickett et al., 2013) . Here, we demonstrate that plumage coloration in female and social male tree swallows is similarly related to nestling immunity while different aspects of coloration related to bactericidal capacity in adults. Bactericidal capacity of nestlings and adult males was also related to their blood selenium concentrations, suggesting that the role of micronutrients other than carotenoids in mediating immunity in wild birds warrants further examination.

In ASY females, we found that the brightness of white breast plumage was positively associated with the bactericidal capacity of her plasma. White brightness may be associated with either the quantity or activity of complement enzymes or lysozyme that are primarily responsible for this aspect of the innate immune response (Matson et al., 2006). The results for females were similar to other recent studies that indicated the size, number, or reflectance of white plumage patches is related to the strength of the immune response or parasite load in female birds (Hanssen et al., 2006; Zanollo et al., 2012; Blanco and Fargallo, 2013). Our results suggest that greater attention should be paid to the reflectance of large, white patches of plumage, which are infrequently examined in most avian studies despite being relatively common.

In male tree swallows, we detected an interactive relationship between male PC1 plumage scores, blood selenium concentrations and plasma bactericidal capacity. The relationship between selenium and bactericidal capacity looked much like an optimal response curve, but the selenium concentration associated with peak bactericidal capacity differed between males with different plumage characteristics. Selenoproteins play a number of important roles in the immune system, such as increasing the proliferation and activity of immune cells, altering gene expression, and

regulating the redox state of the organism (Maggini et al., 2007; Wintergerst et al., 2007; Huang et al., 2012). One possibility is that males with bluer dorsal plumage modulate these processes differently than greener males when provided with supplemental selenium. However, a stronger innate immune response may also impose a cost because its activation leads to the production of free radicals and oxidative stress (Hasselquist and Nilsson, 2012). Thus, a second possibility is that bluer males balance the trade-off between immunity and oxidative stress differently than greener males when provided with supplemental selenium. In collared (*Ficedula albicollis*) and pied flycatchers (*Ficedula hypoleuca)* larger white plumage patches are associated with greater antioxidant capacity and lower oxidative stress than males with smaller patches (Marko et al., 2011; Moreno et al., 2011). This suggests that structural plumage coloration can be associated with measures of oxidative stress and/or antioxidant capacity, though this remains to be addressed in tree swallows.

Our results indicated that the same aspect of adult immunity was related to different types of plumage coloration in male and female tree swallows. We suggest that this results from the different selective pressures acting on males and females. Male coloration is likely maintained by traditional sexual selection where females gain benefits from colorful males (Andersson, 1994). Associations between brighter and/or lower wavelength hues and extra-pair mating success (Bitton et al., 2007), higher survival or site fidelity (Bitton and Dawson, 2008), and a stronger innate immune response (this study) suggest dorsal coloration in male tree swallows is related to indirect, genetic benefits that are passed to offspring. In contrast, female coloration is likely maintained by social selection resulting from competition for breeding and non-breeding resources. One possibility is that brighter white females obtain nest sites

located closer to prime foraging areas and are in better condition and able to mount a stronger immune response as a result. At a proximate level, it is possible that sex differences in the timing of molt (Hemborg, 1999; Dawson, 2009), seasonal hormone concentrations (Dawson, 2009; Small and Schoech, 2015), or in immunity (Hasselquist, 2007) lead to different relationships between coloration and immunity in males and females.

A relationship between white intensity and resource quality or parental care could explain why nestling bactericidal capacity was positively associated with white brightness in both sexes. If white brightness is associated with better resource availability or food quality then offspring may be in better condition and able to devote more resources to development of their immune system. In one model, we detected an interactive effect of nestling residual mass and blood selenium concentrations suggesting that parental provisioning could influence nestling bactericidal capacity. Additionally, blood selenium concentrations were positively associated with nestling bactericidal capacity and with a stronger relationship between social male breast brightness and nestling bactericidal capacity. This again suggests a relationship between nutritional conditions and offspring immunity. Indeed, the high rate of extra-pair mating in tree swallows (Whittingham and Dunn, 2001), makes it unlikely that the relationship between male coloration and offspring immunity is attributable to genetic inheritance alone. This relationship could also be the result of a maternal effect if brighter white females deposit more carotenoids (Saino et al., 2003; Biard et al., 2007), antibodies (Hasselquist and Nilsson, 2009), lysozyme (Board and Fuller, 1974) or testosterone (Clairardin et al., 2011) in eggs, which can all influence the immunocompetence of their offspring. While beyond the scope of the current study,

future work should assign parentage to offspring and employ a cross-fostering design to better disentangle the contributions of genetics, micronutrient availability, and other parental effects on offspring immunity.

Nestlings and bluer adult males showed stronger innate immune responses at elevated blood selenium concentrations, but this was not the case for females. Rapid growth in avian young (Costantini et al., 2006; Alonso-Alvarez et al., 2007) and activation of the immune system (von Schantz et al., 1999; Bertrand et al., 2006; Hasselquist and Nilsson, 2012) are both associated with increased production of reactive oxygen species and/or reduced antioxidant defenses. Given that the generation of reactive oxygen species is associated with reduced survival and fecundity in birds (Vleck et al., 2007; Bize et al., 2008), nestling tree swallows may not be able to afford the combined oxidative cost of rapid growth and development of a robust immune response. However, nestlings with greater blood selenium concentrations may be able to offset the combined oxidative costs of growth and immune system development and activation. Males and females had very similar blood concentrations of selenium, but it may be allocated to tissues differently in each sex. In mammals, there are sex differences in selenium metabolism and expression of selenoproteins in tissues (reviewed in Schomburg, 2012) and in poultry, there are sex differences in selenoenzyme activity in different tissues (Shaaban et al., 2008). Thus, female tree swallows may allocate their selenium to tissues or processes that do not lead to enhanced bactericidal capacity while males do. The sample size for the female analysis was relatively small and further research with more robust sample sizes is necessary to determine if there are sex differences selenium usage in wild birds.

In conclusion, our study indicates that plumage coloration in adult tree swallows is related to their bactericidal capacity and that of their nestlings and that the relationships between plumage coloration and immunity can be influenced by selenium. We found that different aspects of coloration were related to bactericidal capacity in each sex which may ultimately be due to the different selective pressures operating on each sex, sexual selection in males and social selection in females. Indeed, our results are similar to a recent study that found carotenoid-based ornamentation was related to immunity in female American goldfinches (*Spinus tristis*) but not in males (Kelly et al., 2012). While it is becoming increasingly clear that discrete white plumage patches can be related to individual condition (McGlothlin et al., 2007), quality (Hanssen et al., 2006; Hanssen et al., 2009; Zanollo et al., 2012), or offspring quality (Remeš and Matysioková, 2013), very few studies have examined the reflectance of large white plumage areas (but see Bonato et al., 2009; Bonato et al., 2013). Bonato et al. (2013) found a positive relationship between the white brightness of the genetic father and humoral immunity in offspring, a result quite similar to the one we found for innate immunity. Together, this suggests that greater attention should be paid to the reflectance of large, white plumage areas because they may be signals of individual quality. We also found that blood selenium concentrations of adult males and nestlings were positively associated with one aspect of their innate immune response (current study, Beck et al., 2014b). These results suggest that signal honesty may be altered by micronutrient availability, because bluer males only showed greater bactericidal capacity when they also had higher blood selenium concentrations. Studies in other wild and domesticated birds have detected positive relationships between tissue or dietary selenium concentrations and aspects of immunity, though these studies were

focused on selenium supplementation or relatively high concentrations of selenium (Wayland et al., 2002; Brady et al., 2013; Surai and Fisinin, 2014). Our study suggests that more natural variation in selenium bioavailability, within or among populations, could influence the physiology of adults or young. Given the current interest in the regulation of oxidative stress and immunity in studies of behavioral ecology and life history (Hasselquist and Nilsson, 2012), and that selenium can have profound effects on these aspects of physiology (Huang et al., 2012), further examination of its role and that of other inorganic micronutrients in wild populations is warranted.

Materials and Methods

Study Species

Tree swallows are secondary cavity nesters and readily settle in nest boxes. In this population, most tree swallows are single-brooded but are occasionally double brooded as has been found in other southern populations (Monroe et al., 2008). The average clutch size (N = 585 nests) in this population is 5.16 ± 0.03 (range 2-7 eggs) and average brood size 4.80 ± 0.04 (range 1-6). Tree swallows are typically socially monogamous but genetically promiscuous with 80% of broods having mixed parentage in other populations (Whittingham and Dunn, 2001).

Study Area and selenium exposure

We studied a nest box breeding population of tree swallows in Roane (35.9° N, 84.5° W) and Loudon Counties (35.8° N, 84.3° W), TN, USA during the 2011 and 2012 breeding seasons. All of the nest boxes were located within 70 m of the shore along the Emory, Clinch, or Tennessee rivers and were located in 9 colonies that contained 32-86 nest boxes. Boxes were spaced 15 m apart when in a single row, or 20 m apart

with a staggered alignment when there were two or more rows. This study was conducted in an area where tree swallows were exposed to modestly elevated concentrations of selenium from a recently remediated coal fly ash spill and at nearby reference colonies where swallows were exposed to lower background concentrations. Fly ash contains high concentrations of many elements and exposure to fly ash can have severe negative effects on reproduction and physiology in a number of taxa (Rowe et al., 2002; Janz et al., 2010; Rowe, 2014). However, this spill occurred in December 2008 and extensive remediation efforts coupled with natural processes such as off-site transport greatly reduced element exposure well before our study began in 2011. Our previous research on swallows at this site indicated that reproductive success, nestling residual body mass, and nestling stress physiology were not influenced by exposure to residual element contamination and that nestling bactericidal capacity and cutaneous immune responses did not differ among colonies (Beck et al., 2014, Beck et al, 2015). The only element in nestling blood that was modestly elevated above background levels in both years of our research was selenium. Thus, the study site provided the unique opportunity to study the potential positive effects of modest dietary enrichment of selenium in a wild population of birds.

Selenium concentrations were quantified in whole blood of adults and nestlings using Inductively Coupled Mass Spectrometry at the Trace Element Analysis Core at Dartmouth College. A more detailed description of the study area and analytical methods used to quantify selenium concentrations can be found elsewhere (Beck et al., 2014b).

Adult and nestling capture, blood and feather sampling

We checked nest boxes every four days beginning in late March and every 3 days beginning in early June for signs of nesting activity so that we could accurately record clutch initiation and hatching dates and capture parents at appropriate times. We captured adults using mist nets or trapped them in the nest box while incubating or provisioning young. Adults were sexed by the presence of a brood patch (females) or cloacal protuberance (males) or, if these were absent, by measuring the wing length, with wings shorter than 113 mm being indicative of females and wings longer than 122 mm being indicative of males (Stutchbury and Robertson, 1987a). Females were also aged as SY or ASY based on plumage coloration as described previously. Adults were banded with a combination of one metal band and one colored leg band placed on opposite legs to help us distinguish individuals breeding in neighboring boxes and between males and females from a distance. From each adult, we obtained a small blood sample ($\leq$ 120 µl) and measured left and right tarsus length (each was measured twice), wing chord, and body mass. When nestlings were 13 days old, we obtained morphological measurements (as described for adults), banded them with a metal leg band, obtained a small blood sample ($\leq$ 120 µl), and performed the immune challenges.

Quantification of plumage coloration

To quantify plumage coloration of adults, we collected 8-10 feathers each from standardized locations on the back, rump, and breast of all adults. Feathers were taped to pieces of black construction paper with low reflectance from 320-700 nm and in a manner similar to the way they laid on the bird. We quantified plumage coloration

using a JAZ reflectance spectrophotometer (Ocean Optics, Dunedin, Fl) with a 200 micron fiber optic probe. The probe tip was held in a sheath that excluded ambient light and kept the probe tip 5 mm from the sample surface. The spectrometer lamp was allowed to warm up for 10 min prior to making the first measurement. Spectra were produced relative to a white standard (Labsphere, North Sutton, NH) and the spectrometer calibrated to the white standard between samples. All measurements were made perpendicular to the sample surface and we made 4 measurements of each region (back, rump, and breast). We used the program CLR version 1.05 (Montgomerie, 2008) to smooth reflectance spectra and calculate color variables. We calculated brightness of the breast and dorsal plumage coloration as average percent reflectance from 320-700 nm. For dorsal plumage, we calculated hue as the wavelength at which peak reflectance occurred. We also calculated three measures of chroma for dorsal plumage: UV, blue, and green. We calculated chroma as the total reflectance in the UV (320-400 nm), blue (400-510 nm) and green (510-605 nm) divided by the total reflectance from 320-700nm.

Immune response

We examined one aspect of the innate immune response of nestling and adult tree swallows in June and July 2012 by evaluating the bactericidal capacity of plasma (following Liebl and Martin, 2009). This assay reflects the ability of complement enzymes and lysozyme to destroy cell walls and lyse cell membranes of bacteria (reviewed in Matson et al., 2006). Blood samples were obtained from adults trapped in their nest box while provisioning young or from nestlings 13 days post-hatch. The area around the puncture site was cleansed with 70% ethanol prior to blood collection from

the brachial vein. We collected 120 µl of blood in heparinized capillary tubes, 60 µl was used to assess bacterial killing capacity and 60 µl was reserved to quantify selenium concentrations. Samples for the bactericidal assay were centrifuged at 10000 rpm for 5 min within 6 h of collection and the plasma fraction placed in a sterile 0.5mL tube. The bactericidal assay was run with fresh plasma on the day the sample was collected. Samples were run in triplicate by aliquoting 3.5 µl of plasma into sterile tubes and diluted with 31.5 µl of sterile PBS (1:10 dilution). We added 12.5 µl of 10^5 bacteria/ml *Escherichia coli* (ATCC 8739, E^{power} microorganisms; Microbiologics ®St. Cloud, Minnesota) solution to each tube and vortexed each sample. Samples were incubated at 37°C for 30 min and 250 µl of tryptic soy broth (TSB, Sigma Aldrich, St. Louis, Mo) was added to each tube, and samples were incubated for an additional 12 h at 37°C. Positive controls were created in triplicate by adding 12.5 µl 10^5 bacteria/mL to 250 µl of TSB and we created duplicate blanks by combining 50 µl of PBS with 250 µl of TSB. We checked that bacteria were not introduced during bleeding or sample processing by making two additional controls without adding bacteria stock that contained 3.5 µl of plasma, 250 µl of TSB, and 50 µl of PBS.

Following incubation, samples were vortexed and a Nanodrop Spectrophotometer (ND-2000, Thermo Scientific, Pittsburgh, Pa) was used to measure the absorbance of each sample at OD300. The absorbance of each sample and the positive controls were each averaged and used to calculate the proportion of bacteria killed as 1-(sample absorbance/positive control absorbance). The Nanodrop arm was cleansed between each sample with 70% ethanol and the entire work area was cleansed with ethanol before and after each work day.

We also quantified the response of nestlings to PHA (Sigma Aldrich, St. Louis, Mo) by injecting the patagium (wing web) of nestlings with 0.15mg of PHA dissolved in 30 µl phosphate buffered saline (after Smits et al., 1999; Hawley et al., 2009). The injection of PHA leads to localized swelling due to the influx and proliferation of leukocytes and T-cells at the injection site (Martin et al., 2006) and a build-up of free radicals that are produced during phagocytosis by leukocytes (Peretz, 1989). Feathers were first cleared from the wing web and the area sterilized using 70 % ethanol. One individual held the nestling with its right wing extended in a standardized position while a second made all measurements and performed the injections. Prior to and approximately 24 h following injection, the thickness of the wing web was quantified to the nearest 0.01 mm using a micrometer. The micrometer dial was not visible to the measurer while making the measurement. We made 5 pre- and 5 post-injection measurements and discarded the lowest and highest values in each set and used the remaining three to produce average pre- and post-injection widths for each individual. We divided the difference between the post and pre-injection measurements by the pre-injection measurement to adjust for pre-injection differences in wing web width and used this as our measure of the cell-mediated immune response. The average time ± SD between injection and measurement was 24.8 ± 2.3 h and there was no correlation between the time between injection and measurement and the PHA response or the average post-injection swelling (both $r \leq 0.25$, df = 31, $P \geq 0.18$).

Analyses

We used Kolmogorov-Smirnov tests and normality plots to determine if variables met the assumptions of parametric tests. We log transformed continuous

variables (plumage hue and blood selenium concentration) or used arcsine square root transformations for proportion data (plumage chroma and brightness, immune responses). Using data collected in 2011 and 2012, male and ASY female plumage variables were first compared using a MANCOVA to determine if they are sexually dichromatic. For all males and ASY females, the dorsal color variables for blue, structural color were highly correlated and we used principal components analysis (PCA) to reduce the dimensionality of the data set while producing variables that were not linearly related. We used transformed color variables from both years in the PCA and used Varimax factor rotation to simplify the factor loadings. For adults captured in both years, we selected a single year of data to include in the PCA. For white breast plumage, we calculated its average brightness and included it in the analyses as well. We did not perform any analyses with SY females because we had a much smaller sample size for this age class and their plumage coloration is melanin-based (Bentz and Siefferman, 2013) and differs dramatically from the structural coloration of ASY females.

We used correlations to determine if males and ASY females paired assortatively by PC scores rather than the individual color variables to minimize the number of statistical comparisons made. Colonies were classified as exposed to modestly elevated selenium (sites previously impacted by the spill) or as reference colonies with background exposure to selenium (Beck et al., 2014a). Swallows were categorized as adult males, ASY females, or nestlings and we then used 2-way ANOVAs to compare blood selenium concentrations and bactericidal capacity between impacted and reference colonies and between the sexes and nestlings. We used residual body mass (residuals from a regression of mass on tarsus length) as a measure of body

condition. While the use of residuals as a measure of body condition is controversial (Green, 2001), studies have shown that residuals do correlate well with lipid reserves (Ardia, 2005; Schulte-Hostedde et al., 2005) and residual body mass is related to the immune response in tree swallows (Palacios et al., 2012). Using data pooled from all of the colonies, we used linear regressions and backward elimination of non-significant terms to examine the effects of plumage coloration, sample date, residual body mass, and selenium concentrations on the immune responses of adults and nestlings. We allowed terms to remain in the model as long as $p \leq 0.10$ but only considered their contribution to be statistically significant when $p \leq 0.05$. We used the percent bacteria killed and wing web swelling as dependent variables. We evaluated the response to PHA injection and bactericidal capacity in a single nestling from each box to avoid issues with pseudoreplication. We ran separate models to examine relationships for males and females to determine how adult phenotype related to their bactericidal capacity. We also ran separate models for male and female parents when examining nestling immune responses for two reasons. First, males and females pair assortatively by coloration (see results), and including their color variables simultaneously in the model introduces issues with multicollinearity. Second, we caught only a single parent at some boxes and limiting our analysis to nests where both parents were captured would significantly reduce sample sizes. Sample sizes varied slightly among tests due to incomplete sampling of individuals (*e.g.*, failure to collect feathers for spectroscopy). All statistical tests were two-tailed with $\alpha = 0.05$. All statistical analyses were performed using PASW 18 (SPSS, 2009).

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Figures

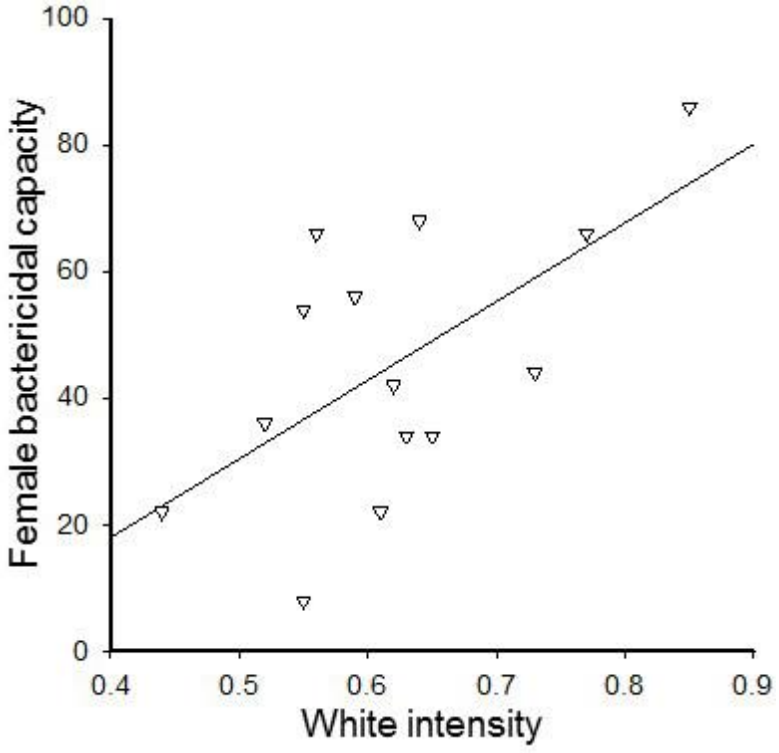

Figure 1. The relationship between ASY female plumage coloration and her bactericidal capacity. We detected a significant positive relationship between the brightness of white breast plumage and the bactericidal capacity of female plasma in ASY females ($r^2 = 0.31$, $df = 11$, $p = 0.05$).

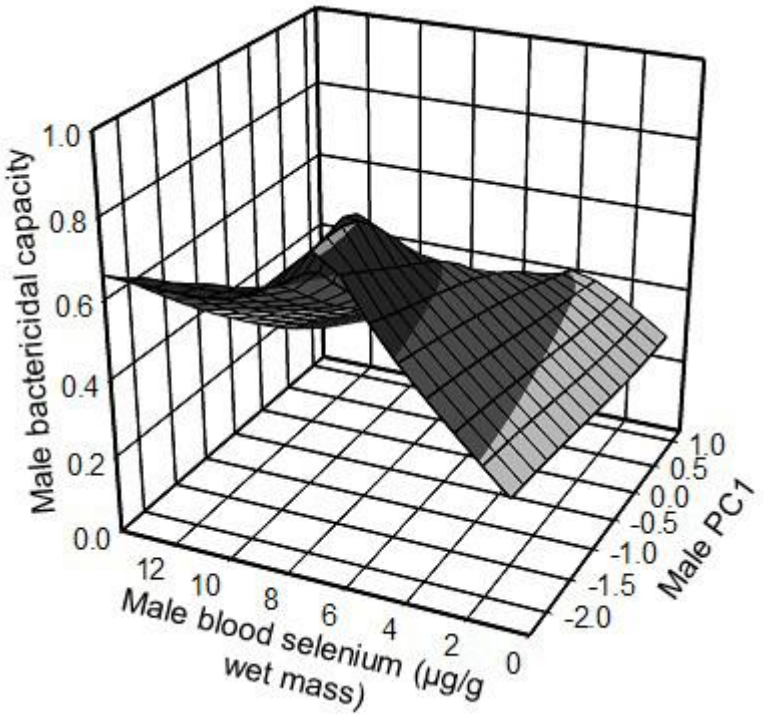

Figure 2. Relationships between male dorsal plumage coloration, blood selenium concentrations and bactericidal capacity. Lower PC1 scores (greater blue and uv chroma, lower hue) were associated with greater bactericidal capacity in males with greater blood selenium concentrations. However, greater PC1 scores (greater green chroma and hue) in combination with greater blood selenium concentration were associated with lower bactericidal capacity in males. Shades of gray denote changes in bacterial killing capacity of 0.1%.

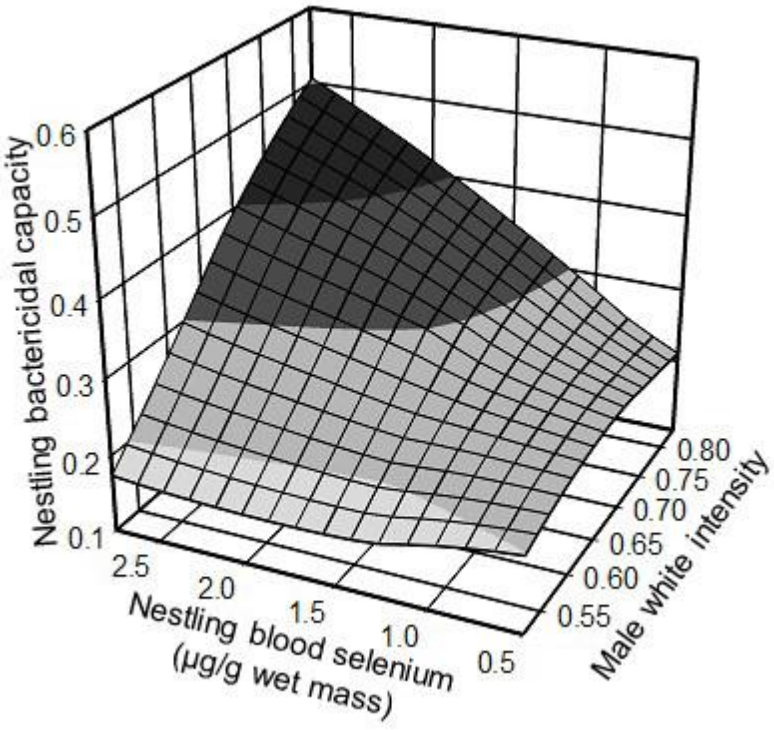

Figure 3. Relationships among (A) female white breast brightness and nestling residual bactericidal capacity and (B) the interactive relationship between nestling residual body mass and blood selenium concentrations and nestling residual bactericidal capacity. Our final regression model included nestling blood selenium concentrations, sample date, white breast brightness, nestling condition, and the interaction between nestling condition and blood selenium concentrations. We took the residuals of a regression between sample date and bactericidal capacity and repeated the backward linear regression and all of the terms remained in the final model. (A) Female white brightness remained positively related to residual bacterial killing capacity. (B) Nestlings with low residual body mass and low blood selenium

concentrations had the lowest bactericidal capacity while nestlings with greater blood selenium concentrations and either low or high residual body mass had the greatest bactericidal capacity Shades of gray denote changes in residual bacterial killing capacity of 0.1%.

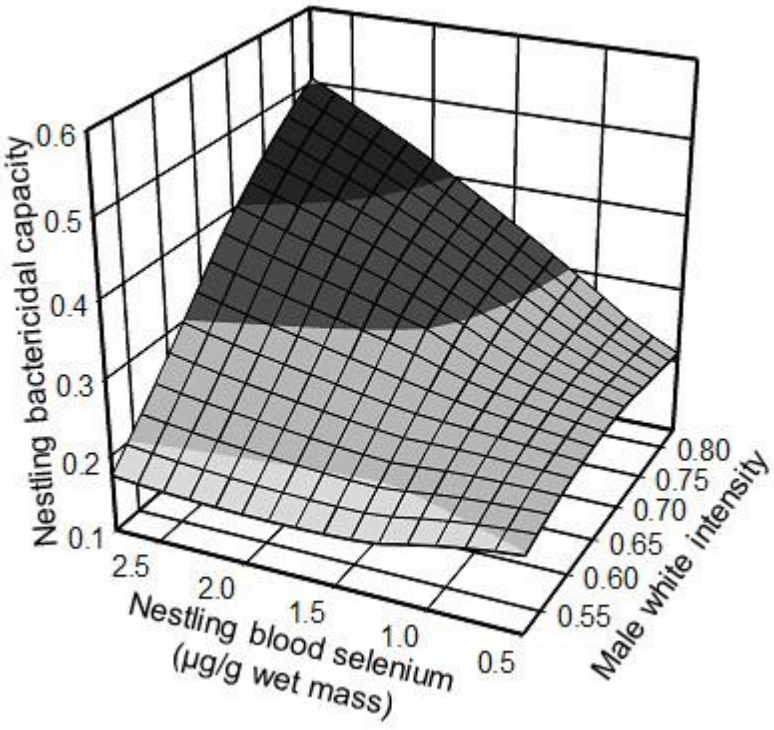

Figure 4. Relationships between male white breast brightness, nestling blood selenium concentrations, and bactericidal capacity of nestling plasma. Nestlings with greater blood selenium concentrations had greater bactericidal capacity when the male attending the nest had brighter white ventral plumage, but not if the social male had less intense breast coloration. Shades of gray denote changes in bacterial killing capacity of 0.1%.

Tables

Table 1. Univariate means and standard errors for plumage coloration of adult tree swallows. Chroma and hue were calculated for the dorsal plumage.

Color parameter	Male	ASY Female
White brightness	0.64 ± 0.01	0.64 ± 0.01
Dorsal brightness	0.16 ± 0.002	0.16 ± 0.003
Green chroma	0.30 ± 0.002	0.32 ± 0.003
Blue chroma	0.39 ± 0.003	0.36 ± 0.003
UV chroma	0.14 ± 0.002	0.13 ± 0.002
Hue	483.1 ± 4.0	501.4 ± 4.8

Table 2. Factor loading matrix from principal components analysis of adult male and ASY female dorsal, blue plumage.

Color variable	PC1	PC2
Blue brightness	0.036	0.944
Green chroma	0.967	0.164
Blue chroma	-0.812	0.409
UV chroma	-0.871	-0.274
Hue	0.964	-0.148
Eigen value	3.28	1.18
% variance	65.7	23.6

Table 3. Mean (± standard error) blood selenium (Se) concentrations (μg/g wet mass) and bactericidal capacity (BKA) of adult and nestling tree swallows. Exposed colonies have greater Se bioavailability due to a fly ash spill than reference colonies.

Sex/Age	Se Exposed	*N*[a]	Se Reference	*N*	BKA Exposed	*N*	BKA Reference	*N*
Male	4.82 ± 0.27	20	2.44 ± 0.40	9	0.47 ± 0.03	20	0.37 ± 0.05	9
Female	4.84 ± 0.54	5	2.02 ± 0.38	10	0.36 ± 0.07	5	0.48 ± 0.05	10
Nestling	1.41 ± 0.18	44	0.87 ± 0.21	32	0.26 ± 0.02	44	0.25 ± 0.03	32

[a] *N* Sample size for each group

Table 4. Full and final models examining the relationship between bactericidal capacity and plumage coloration of adult tree swallows. In cases where no terms contributed significantly to the model, we present results from the penultimate model.

Term	**β**	***P***
ASY female full model r^2 = 0.84, df = 2, P = 0.58		
PC1	-0.142	0.46
PC2	0.043	0.73
White brightness	-0.227	0.22
Condition	-0.287	0.166
Sample date	0.091	0.89
Selenium	2.669	0.21
PC1 x selenium	0.018	0.98
PC2 x selenium	1.066	0.38
White brightness x selenium	-16.956	0.20
Condition x selenium	-2.269	0.20
ASY female final model r^2 = 0.31, df = 11, P = 0.05		
White brightness	1.248	0.05
Male full model r^2 = 0.54, df = 13, P = 0.23		

PC1	-0.017	0.71
PC2	-0.060	0.31
White brightness	0.406	0.49
Condition	-0.045	0.44
Sample date	-0.001	0.77
Selenium	0.080	0.90
PC1 x selenium	-0.808	0.06
PC2 x selenium	0.034	0.92
White brightness x selenium	-1.555	0.66
Condition x selenium	0.170	0.56
Male final model r^2 = 0.42, df = 20, P = 0.01		
PC1	-0.034	0.33
Selenium	0.078	0.61
PC1 x selenium	-0.823	0.004

Table 5. Full and final models examining the relationship between parental plumage coloration and bactericidal capacity of nestling tree swallows. In cases where no terms contributed significantly to the model, we present results from the penultimate model.

Term	**β**	***P***
ASY female full model r^2 = 0.53, df = 26, P = 0.01		
PC1	-0.021	0.22
PC2	0.032	0.20
White brightness	0.450	0.06
Nestling condition	0.005	0.57
Sample date	0.009	0.01
Selenium	0.140	0.18
PC1 x selenium	0.078	0.42
PC2 x selenium	0.169	0.433
White brightness x selenium	1.017	0.54
Nestling condition x selenium	-0.072	0.09
ASY female final model r^2 = 0.45, df = 31, P = 0.002		

White brightness	0.460	0.05
Nestling condition	0.004	0.60
Sample date	0.010	0.003
Selenium	0.205	0.03
Nestling condition x selenium	-0.083	0.04
Male full model r^2 = 0.40, df = 40, *P* = 0.02		
PC1	-0.011	0.61
PC2	-0.005	0.83
White brightness	0.475	0.03
Nestling condition	0.007	0.29
Sample date	0.001	0.89
Selenium	0.068	0.46
PC1 x selenium	0.106	0.42
PC2 x selenium	-0.149	0.31
White brightness x selenium	3.353	0.002
Nestling condition x selenium	-0.064	0.13
Male reduced model r^2 = 0.30, df = 51 *P* = 0.004		

White brightness	0.449	0.02
Selenium	0.090	0.25
White brightness x selenium	2.951	0.002

Table 6. Full and final models examining the relationship between parental plumage coloration and the response to PHA injection in nestling tree swallows. In cases where no terms contributed significantly to the model, we present results from the penultimate model.

Term	**β**	***P***
ASY female full model r^2 = 0.37, df = 6, P = 0.93		
PC1	0.028	0.85
PC2	0.045	0.80
White brightness	-0.231	0.85
Condition	0.007	0.90
Sample date	0.005	0.93
Selenium	0.433	0.61
PC1 x selenium	1.368	0.34
PC2 x selenium	-0.217	0.86
White brightness x selenium	-2.964	0.76
Nestling condition x selenium	0.071	0.77
ASY female final model r^2 = 0.05, df = 15, P = 0.38		
Selenium	0.403	0.38

Male full model r^2 = 0.41, df = 8, P = 0.87		
PC1	0.087	0.64
PC2	0.087	0.70
White brightness	0.275	0.91
Condition	0.063	0.45
Sample date	-0.011	0.92
Selenium	-0.502	0.54
PC1 x selenium	2.494	0.12
PC2 x selenium	-1.744	0.25
White brightness x selenium	8.605	0.49
Nestling condition x selenium	-0.984	0.12
Male reduced model r^2 = -0.05, df = 17, P = 0.73		
Selenium	-0.205	0.73