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Elizabeth C. Sassani
Boise State University

Christeena Sevy
College of Idaho

Erin H. Strasser
Boise State University

Alexandra M. Anderson
Boise State University

Julie A. Heath
Boise State University

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Elizabeth C. Sassani
Boise State University

Alexandra M. Anderson
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College of Idaho

Julie A. Heath*
Boise State University
julieheath@boisestate.edu

Erin H. Strasser
Boise State University

Abstract

In wild birds, the proximate and ultimate factors that affect circulating carotenoid concentrations remain poorly understood. We studied variation in plasma carotenoid concentrations across several scales: annual, seasonal, pair, territory and individual, and evaluated whether carotenoid levels explained reproductive outcome of wild American kestrels (*Falco sparverius*). We sampled plasma carotenoid concentrations of 99 female and 80 male incubating kestrels from April-June in 2008-2012. Plasma carotenoid concentrations were explained by an interaction between year and sex, date, and random effects for pair and individual identity. In general, plasma carotenoid concentrations of males were significantly higher than females, but this depended on year. Within a breeding season, earlier nesting kestrels had higher carotenoid concentrations than later nesting kestrels, a pattern that is coincident with seasonal trends in local fitness. Pair and individual identity explained variation in carotenoid concentrations suggesting that carotenoid concentrations of mated birds were correlated, and some individuals consistently maintained higher carotenoid levels than others. Male carotenoid concentrations were positively associated with number of young fledged per pair. These results are consistent with the hypothesis that higher quality individuals have higher carotenoid levels compared to lower quality individuals, despite annual variations in carotenoid availability.

Additional Keywords: date, integument, mate, nest success, productivity, quality, sexual selection, size, territory

Introduction

Carotenoids are the primary pigments responsible for producing red, orange, and yellow colors in bird feathers and skin. Also, carotenoids support immune function (Blount *et al.*, 2003; McGraw & Ardia, 2003; McGraw, Nolan, & Crino 2011) and act as antioxidants (Simons, Cohen & Verhulst, 2012). Birds must acquire carotenoids through their diet because carotenoids cannot be synthesized *de novo* (Brush, 1981). Acquisition of dietary carotenoids can be limited by environmental conditions that reduce primary productivity (Slagsvold & Lifjeld, 1985) or the absence of prey items with high carotenoid content (Negro *et al.* 2000). In addition, endogenous factors such as hunting ability (Bortolotti *et al.*, 2000), hormone concentrations (Blas *et al.* 2006), genetics (Vergara *et al.* 2015), sex (McGraw *et al.*, 2003), or infection can affect carotenoid acquisition, assimilation, or utilization. For example, presence of endoparasites can lower carotenoid absorption from the gut (Hörak *et al.* 2004) or lower circulating plasma carotenoid concentrations (Biard *et al.*, 2010). If carotenoid availability is limited by environmental or endogenous factors, there may be a trade-off between the utilization of carotenoids for integument color and an individual's ability to cope with adverse conditions (Olson & Owens, 1998; Tschirren, Fitze & Richner, 2003). Because of this trade-off, researchers have hypothesized that carotenoid-based coloration acts as an honest signal of individual quality (Olson & Owens, 1998). This hypothesis has been supported by studies showing positive associations between adult carotenoid-based coloration or adult plasma carotenoid concentrations and the provisioning of offspring (Casagrande *et al.* 2006, Linville, Breitwisch & Schilling, 1998), nestling health (Hidalgo-Garcia 2006), and number of young produced (McGraw *et al.* 2001, Safran *et al.* 2011). Plasma carotenoid concentrations typically reflect dietary supplementation (Alonso-Alvarez *et al.*, 2004;

Casagrande *et al.*, 2007) or immune activation (Pérez-Rodríguez *et al.*, 2008; Biard *et al.*, 2009) and are positively associated with integument color (Bortolotti *et al.*, 1996, although see Blas *et al.*, 2013). Consequently, studies of circulating carotenoid concentrations in plasma are useful for understanding the environmental and physiological factors associated with variation in carotenoid concentrations and how this variation relates to fitness correlates, such as productivity.

American kestrels (*Falco sparverius*) have carotenoid-based skin coloration in their ceres, lores, and legs, and hue corresponds with plasma carotenoid concentrations (Bortolotti *et al.*, 1996; Bortolotti *et al.*, 2000). Previous research suggests that both exogenous and endogenous factors play a role in kestrel carotenoid acquisition and utilization. In a study of non-breeding, wild kestrels, male carotenoid-based skin color was positively associated with territory quality (Bostrom & Rishardson, 2006). In captive kestrels, plasma carotenoid concentrations changed over the course of the breeding cycle, independent of changes in diet, with higher concentrations during mate selection and egg laying that decreased through the nestling stage (Negro *et al.*, 1998). Whether kestrel plasma carotenoid concentrations are associated with productivity has not been determined.

Our objective was to study the proximate and ultimate correlates of American kestrel plasma carotenoid concentrations. We examined variation in carotenoid concentrations between years and within seasons because, in our southwestern Idaho study area, kestrel productivity varies annually and seasonally. Specifically, productivity averages as many as 3.5 young fledged per pair in 'good' years and as low as 1.3 young fledged per pair in 'poor' years (Steenhof & Peterson, 2009). Within a breeding season, kestrels show a broad range of nest initiation dates, from early March – July, and nest initiation dates are associated with local fitness. Earlier nesting birds are more likely to produce local recruits compared to later nesting kestrels (Steenhof & Heath, 2013) and earlier nesting females have higher apparent survival than later nesting females (Steenhof & Heath, 2009). We predicted that plasma carotenoid concentrations would have high inter-annual variation, coincident with annual patterns in reproductive success. We predicted either a seasonal increase in plasma carotenoids concentrations if plasma carotenoid levels were related to prey availability, or a seasonal decrease in plasma carotenoid concentrations if carotenoid levels reflected local fitness. By sampling individuals and territories over several years, we explored whether plasma carotenoid concentrations were consistent within an individual or within specific territories. In addition, we examined whether individual carotenoid concentrations were correlated with body mass or the carotenoid concentrations of their mates. Finally, we determined whether male or female plasma carotenoid concentrations explained variation in number of young fledged per pair (productivity) or the mass of young at fledging. We predicted that plasma carotenoid concentrations would be positively associated with productivity and mass of young.

Material and Methods

From 2008 to 2012, we monitored American kestrel nest boxes mounted on posts or trees in rural and suburban areas in southwestern Idaho, USA. Each nest box was considered a territory as no more than one pair bred in a nest box at one time and nesting kestrels defended nest boxes from other kestrels. Beginning in early March, we visited boxes every 7–10 days to determine occupancy and clutch status. For each nest check, we plugged the entrance to the nest box to capture the adult and checked clutch size. Kestrels typically laid one egg every other day until the clutch was complete (4–6 eggs). If we discovered a nest with 5–6 eggs, we removed the incubating adult for sampling. If we discovered a nest with < 5 eggs we estimated the number of days until the clutch could have 6 eggs $[(6 - \text{number of eggs}) * 2]$ and returned at that time to capture and sample the adult. We used the same calculation to estimate the clutch initiation date by back dating 2 days for every egg in a clutch. We collected approximately 0.4 ml of blood from the jugular vein with a 26½ gauge needle and syringe and stored samples in heparinized vials placed on ice until we returned to the lab. We sexed kestrels based on plumage, collected morphological measurements, and marked birds with U.S.G.S. aluminum bands or recorded bands of previously marked birds, and then placed the bird back in the nest box in < 15 mins. We returned to the nest box every 1-2 days to capture the mate of the sampled bird. We limited our sampling to incubating birds because they were relatively easy to capture, and we wanted to reduce variation in carotenoid concentrations associated with different breeding stages (Negro *et al.*, 1998). After capturing adults, we returned to nest boxes near estimated hatch dates and again when young were ~ 25 days old to measure and mark the young birds. We excluded pairs known to be re-nesting from our analysis.

In the lab, blood samples were centrifuged at 10,000 rpm for 15 minutes to separate the plasma from the cellular fraction. Plasma was stored at -80°C until analysis. For carotenoid concentration analysis, 20 µl of plasma was combined with 380 µl of acetone and centrifuged at 1500 rpm for 10 minutes. The supernatant was removed and placed in individual cuvettes and capped with acetone resistant caps. Following Bortolotti *et al.* (1996), absorbance levels were read at 476 nm by spectrophotometer, and carotenoid concentrations were estimated based

on a standard curve for lutein (alpha-Carotene-3,3'-diol, Sigma-Aldrich, PA), the predominate carotenoid in kestrel plasma (Bortolotti *et al.*, 2000, Casagrande *et al.*, 2006). All procedures were conducted with approval from Boise State University's Institutional Animal Care and Use Committee (protocol #006-08-007).

Statistical Analysis

We used a linear mixed effects-model with sex, year, and capture date as fixed effects and identities of individuals, territories, and pairs as random effects to predict plasma carotenoid concentrations. We included terms for interactions between sex and year, and sex and capture date. Year was treated as a categorical variable. Capture date was standardized within each year by subtracting the annual mean capture date from each observation and dividing by the standard deviation to facilitate comparison of effect sizes. The natural log of plasma carotenoid concentrations was used to meet assumptions of randomly distributed residuals. Multicollinearity ($|r| > 0.7$) among the predictor variables was checked with a correlation analysis, and there were no significant correlations. Non-significant terms from the full model were removed in a stepwise fashion until all term P 's < 0.05 . All fixed parameter estimates were based on the final model. We tested whether the variance of random effects was 0 with restricted likelihood ratio tests (package RLRsim). We used *a posteriori* least-squared means to compare means between and within sexes across all years.

We used a linear mixed model with mass as a fixed variable and individual identity as a random variable to examine the association between body condition and carotenoid concentrations. We used body mass as an estimate of "condition" because size-corrected mass indices have not been validated for American kestrels (Heath *et al.* 2011). We used a spearman correlation to examine if plasma carotenoid concentrations of paired males and females were correlated. In addition, we explored whether the carotenoid concentrations of males, or females, were predictive of the difference between the plasma carotenoid concentrations of paired males and females using a linear model.

The timing of clutch initiation is associated with kestrel nest survival (Brown *et al.*, unpub data) so we generated residuals from a generalized linear model of number of young fledged per pair predicted by clutch initiation date to account for the effect of timing on the number of young. We used a zero-inflated, negative binomial distribution for this model because number of young were count data and the variance was greater than the mean (not a Poisson distribution). We generate residuals from a model with date instead of including clutch initiation date as a covariate in the same model as plasma carotenoid concentrations because, for kestrels, these variables covary and doing so would inflate error estimates. We used a linear mixed model with adult plasma carotenoid concentrations as a fixed effect and individual identity as a random effect to explain the residual number of young fledged per pair corrected for clutch initiation date. Also, we used a linear mixed model with adult plasma carotenoid concentrations, nestling age (in days) and nestling sex as fixed effects, and individual and brood identities as random effects to explain nestling mass. For these analyses, we created separate models for adult males and females because outcomes of paired birds were not independent. All analyses were performed with R, version 3.0.1 (R Core Team, 2013). Descriptive statistics are mean \pm standard deviation.

Results

We captured 179 incubating American kestrels (female $n = 99$, male $n = 80$) over the course of 5 breeding seasons from 2008 - 2012. Twenty-six birds were sampled in > 1 yr (female $n = 11$, male $n = 15$), producing a total of 212 samples for analysis. Of these samples, 120 were from the adult male and female of the same pair ($n = 60$). Birds sampled in more than one year typically changed nest boxes and mates between years. Samples were collected from kestrels at a total of 77 different nest boxes, with 35 nest boxes being sampled in > 1 yr. Male plasma carotenoid concentrations averaged $25.1 \mu\text{g ml}^{-1} \pm 14.4$ (range: 3.5 - 63.6), and female plasma carotenoid concentrations averaged $16.6 \mu\text{g ml}^{-1} \pm 9.3$ (range: 3.9 - 71.6). Number of young per pair ranged from 0 to 6 with a mean of 2.5 ± 2.1 .

A model with an interaction between sex and year, capture date, and random effects for individual identity and pair best explained plasma carotenoid concentrations (Table 1). In general, plasma carotenoid concentrations of male kestrels were higher than plasma carotenoid concentrations of females but this relationship depended on year (Figure 1). There was no significant interaction between capture date and sex (Wald $\chi^2 = 2.21$, $df = 1$, $P = 0.14$), but there was a significant effect of capture date, with higher plasma carotenoid concentrations in birds nesting earlier in the breeding season and lower plasma carotenoid concentrations in birds nesting relatively later in the breeding season ($\beta = -0.11$, CI: -0.16 - -0.05, Table 1, Figure 2). The random effects for individual identity and pair explained a significant amount of variation (Table 1). However, the amount of variation explained by the random effect of territory identity was not significantly different from 0 (LRT = 0.15, $P = 0.33$).

Male mass ranged from 92 – 128 g and was associated negatively with plasma carotenoid concentrations ($\beta = -0.22$, CI: -0.44 - -0.02, Wald $\chi^2 = 4.33$, df = 1, $P = 0.03$). Female mass ranged from 104 – 174 g and there was no relationship between mass and plasma carotenoid concentrations (Wald $\chi^2 = 1.72$, df = 1, $P = 0.18$). Plasma carotenoid concentrations of paired males and females were correlated positively ($r_s = 0.48$, $P < 0.001$). Concentrations of carotenoids in males were associated with the difference between carotenoid concentrations of paired males and females ($\beta = 0.79$, CI: 0.67 - 0.91, $F_{1,58} = 166$, $P < 0.001$, Figure 3), suggesting that as male carotenoid concentrations increased the difference between paired individuals increased. Concentrations of carotenoids in females were not associated with the difference between paired male and female carotenoid concentrations ($F_{1,58} = 0.78$, $P = 0.28$).

Male plasma carotenoid concentrations were positively associated with the number of young produced ($\beta = 0.09$, CI: 0.01-0.17, Wald $\chi^2 = 4.31$, df = 1, $P = 0.04$), but there was no significant association between female plasma carotenoid concentrations and the number of young produced ($\beta = 0.10$, CI: -0.02-0.22, Wald $\chi^2 = 2.45$, df = 1, $P = 0.12$). Mass of young male and female kestrels ranged from 92 - 156 g and nestlings were measured from 20 – 26 days of age. Neither male nor female plasma carotenoid concentrations were associated with the mass of their young (male: Wald $\chi^2 = 2.56$, df = 1, $P > 0.1$, female: Wald $\chi^2 = 0.01$, df = 1, $P > 0.9$).

Discussion

Plasma carotenoid concentrations of incubating American kestrels varied by year and sex and declined throughout the season with earlier nesting birds having higher plasma carotenoid concentrations than later nesting birds. Individual identity explained a significant amount of variation in plasma carotenoid concentrations suggesting that some individuals maintained relatively higher plasma carotenoid concentrations than others. Together, these inter- and intra-annual patterns suggest that plasma carotenoid concentrations reflect conditions at several scales: large annual patterns, within-season declines, and individual differences. In addition, plasma carotenoid concentrations of males were positively associated with the number of young produced per pair. This result is consistent with other studies that have demonstrated positive relationships between carotenoid measures and productivity (McGraw *et al.*, 2001; Safran *et al.*, 2010).

We found that in some years, but not others, male kestrels in southwestern Idaho had higher concentrations of plasma carotenoids than female kestrels. Previous research on free-living kestrels in Canada found that males had higher carotenoid concentrations than females in each year of their study (Bortolotti *et al.*, 2000). The inconsistency in sexual differences across studies may be the result of differences in study areas or habitats, or differences in the number of years studied (2 vs. 5 in this study). Isaksson, Von Post & Andersson, (2007) found a significant interaction between sex and year in their study of adult great tit (*Parus major*) plasma carotenoid concentrations. In addition, Eeva *et al.* (2012) and Negro *et al.* (2000) found annual differences in great tit nestling and white stork (*Ciconia ciconia*) plasma carotenoid concentrations, respectively. Factors that affect dietary carotenoid availability may drive annual sex-differences in carotenoid concentrations. For example, in years with lower primary productivity, carotenoid availability may be limited, resulting in similar carotenoid concentrations for males and females (Isaksson *et al.*, 2007). We found that carotenoid concentrations of paired males and females were correlated positively; however, differences between paired adults were predicted by male carotenoid concentrations. These results support the hypothesis that carotenoid availability may limit carotenoid concentrations of males and, in those years, males and females may have similar concentrations. This idea is further supported by a laboratory study where captive male kestrels fed a carotenoid-supplemented diet maintained significantly higher concentrations of plasma carotenoid compared to females (Bortolotti *et al.*, 1996). How annual patterns in sex-biased differences in plasma carotenoid concentrations (and presumably carotenoid-based colors) affect mate selection and communication would be an interesting topic for future research.

Regardless of sex or year, kestrels nesting later in the season had significantly lower plasma carotenoid concentrations than earlier breeding birds. In previous research on seasonal changes in plasma carotenoid concentrations there have been confounding changes in reproductive behavior that also affect carotenoid levels (Hill 1995; Negro *et al.*, 2001; del Val, Negro & Senar, 2013). By only sampling incubating birds, we controlled for the effects of reproductive behavior and found an effect of date on carotenoids concentrations. It seems unlikely that this seasonal pattern in carotenoid levels is positively related to primary productivity or prey availability because, in southwest Idaho, vegetation growth (measured by normalized difference vegetation index, NDVI) and prey (mammals and insects) availability increased from March through May (Smith SH & Heath JA *unpub data*) while kestrel plasma carotenoid concentrations were decreasing. In this population, early-nesting birds have higher local fitness (Steenhof & Heath, 2009, 2013) and higher plasma carotenoid concentrations in these birds would be consistent with the hypothesis that higher quality individuals maintain higher circulating carotenoid concentrations (Safran *et al.*, 2010). Alternatively, migratory status may affect carotenoid

concentrations. Early-nesting kestrels are more likely to be residents and later nesting kestrels tend to be migratory (Anderson *et al.*, In review). Residents and migrants may have different access to dietary carotenoids, have different levels of oxidative stress, or face different immune challenges.

It is often difficult to tease apart the effects of individual characteristics from territory characteristics because “quality” individuals tend to obtain the best territories. By repeatedly sampling the same individuals at different territories, and different individuals at the same territories, we found that individual identity explained significantly more variation than territory identity. Variation explained by individual identity may be related to individual hunting styles, genetic or other inherent differences, or a mix of factors. Kestrels are generalist predators and regularly consume insects, lizards, birds, and mammals. However, individual birds can develop specific hunting styles resulting in different diets between neighboring individuals (Costantini *et al.*, 2005). Individual hunting style and skill could also contribute to the significant amount of variation explained by the random variable for pair identity, and the positive correlation between carotenoid concentrations of mated birds, because male kestrels provision females throughout the pair-bonding, egg-laying, and incubation periods and male hunting style and provisioning capacity could affect female carotenoid concentrations.

Plasma carotenoid concentrations of males were positively associated with the number of young produced per pair. This effect was apparent even after controlling for the effect of clutch initiation date on productivity. Unlike female birds that deposit carotenoids into eggs (Biard, Surai & Møller, 2005; Eeva *et al.*, 2012), it is unlikely that circulating carotenoid concentrations of males directly influence the growth and survival of nestlings. More likely, male plasma carotenoid concentrations are indicative of conditions that affect productivity such as provisioning rate or individual hunting ability (Casagrande *et al.*, 2006). Male kestrels with higher carotenoid concentrations had lower body mass than males with lower carotenoid concentrations which may suggest that males were provisioning mates at a cost to their own energetic demands or that smaller males had higher carotenoid concentrations. These results are seemingly contrary to other studies that have found that birds in better condition or health maintain higher concentrations of carotenoids than birds in poorer condition (Vergara & Fargallo, 2011). However, maintenance of exogenous energy reserves during the breeding season may not be the best strategy for male kestrels. For several raptor species with reverse sexual size dimorphism, smaller males are selected more often by females, have superior hunting skills, and produce more young compared with larger males (Hakkarainen *et al.*, 1996, Pérez-Camacho *et al.*, 2015). Male kestrels may benefit from maintaining lower body mass, higher carotenoid concentrations, and provisioning the female. In addition, if size and carotenoid-based skin color plays a role in kestrel sexual selection, smaller males with higher carotenoid concentrations, and presumably more orange-colored skin, may be able to attract higher quality females compared to larger males with lower carotenoid concentrations. Quality females in may be another contributing factor to higher productivity in males with higher carotenoid concentrations.

We did not find a significant relationship between female plasma carotenoid concentrations and number of young produced or an association between male or female carotenoid concentrations on mass of young at fledging. These results suggest that carotenoid concentrations may not be indicative of female quality. Alternatively, unaccounted for variance in female carotenoid levels compared with male carotenoid levels may have reduced our power to detect a relationships with number of young per pair or mass of young because female carotenoid concentrations can be affected egg-laying (Blas *et al.*, 2013). Future experiments that control for the number of days since clutch completion may find that female carotenoid concentrations are predictive of number of young or condition of young.

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Table 1. Analysis of plasma carotenoid concentrations of incubating American kestrels in southwestern Idaho from 2008-2012. A non-significant interaction term for capture date and sex was removed from the full model. All other terms were significant at $\alpha = 0.05$.

Fixed Variable	Wald χ^2	df	<i>P</i>
Sex	38.68	1	<0.001
Year	82.27	4	<0.001
Capture Date	11.93	1	<0.001
Sex*Year	24.12	4	0.001
Random Variable	LRT		
Individual Bird	4.78		0.009
Pair	5.87		0.007

Figure Legend

Figure 1. Least-squared means and standard errors for plasma carotenoid concentrations of male and female American kestrels incubating eggs in southwestern Idaho, USA. In 2008 and 2009, males had significantly higher carotenoid concentrations than females (*), but in all other years males and females were not significantly different. Both male and female kestrels had higher plasma carotenoid concentrations in 2008 than in other years. Within-sex differences between years are represented by letters, males in capital, females in lower case. Means with different letters were significantly different. Samples sizes are noted next to each symbol.

Figure 2. American kestrel carotenoid concentration residuals, from a model with sex, year, sex*year and individual and pair, across standardized capture date. Kestrels that nested earlier in the breeding season had higher carotenoid concentrations compared to birds that nested later in the breeding season. Dashed lines represent 95% confidence intervals.

Figure 3. The difference between plasma carotenoid concentrations of paired male and female American kestrels and the plasma carotenoid concentrations of the male American kestrels. The difference between paired birds and male carotenoid concentrations were positively correlated suggesting that differences between paired birds were associated with increases in carotenoid concentrations of the males. There was no relationship between the difference between plasma carotenoid concentrations of paired birds and carotenoid concentrations of females.



