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Genetics, local environment and health as factors influencing plasma carotenoids in wild American kestrels (*Falco sparverius*)

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Carotenoids are important as pigments for bright coloration of animals, and as physiologically active compounds with a wide array of health-related functions. Carotenoid-dependent coloration may have evolved as a signal to conspecifics; however, factors that may limit availability of carotenoids are poorly known. We investigated how the acquisition of carotenoids may be constrained by availability in the environment, diet, genetic make-up and health status of wild American kestrels (*Falco sparverius*). Plasma concentrations of siblings at the time of fledging showed a high degree of resemblance; however, a cross-fostering experiment revealed that variance was largely explained by nest of rearing, rather than nest of origin, thus indicating a low genetic component. A multivariate analysis of attributes of nestlings (sex, size, plasma proteins, immune function), parental reproduction (laying date, clutch size) and rearing conditions (brood size, size hierarchy, nestling mortality) showed only a small significant effect of leucocyte differentials on carotenoid concentrations of nestlings. A strong environmental effect on plasma carotenoids was demonstrated by levels of adult kestrels being correlated within mated pairs, and having a significant association with the abundance of voles, the primary prey species, per territory.

Keywords: carotenoids; diet; heritability; immune function; American kestrel

1. INTRODUCTION

Carotenoids are the pigments responsible for many of the brilliant red, orange and yellow colours of animals (Goodwin 1984; Brush 1990). In addition, many are known to be important to health as they function as antioxidants, precursors for vitamin A, and to facilitate immune function (Bendich 1989; Chew 1993; Lozano 1994; Camplani *et al*. 1999). Carotenoids are synthesized *de novo* only in plants, so ultimately animals depend on dietary sources of these pigments for their colour. It is well known in zoo husbandry that inadequate access to carotenoids often results in a loss of coloration (Brush 1981). Similarly, dietary application of carotenoids has been the mainstay of the poultry industry to satisfy consumer demand for richly coloured produce (Fletcher 1992).

While variation in access to pigments has its practical application, it also figures prominently in recent investigations into the evolution of conspicuous coloration, especially sexual dichromatism (Bortolotti *et al*. 1996; Gray 1996). Bright colours may be sexually selected traits that operate as social signals (Lozano 1994; Gray 1996). Such a function depends on pigments, or colour expression, being costly or at least limited to only some individuals; however, to date there has been little consensus, and fewer empirical data, as to whether or not carotenoids are a scarce resource (Hudon 1994; Olson & Owens 1998; Grether *et al*. 1999).

Access to carotenoids may be limited by environmental factors that determine the abundance of the molecules themselves, or by what foods the animal selects. There is abundant indirect evidence of the importance of dietary concentrations of carotenoids in determining coloration or plasma carotenoids in wild birds (Slagsvold & Lifjeld 1985; Hill *et al*. 1994; Linville & Brietwisch 1997; Eeva *et al*. 1998; Negro *et al*. 2000). However, it does not follow that dietary effects also prove that carotenoids in the environment are limited (Hudon 1994). Only Grether *et al*. (1999) have shown that carotenoid availability in the wild limits the expression of colour in animals, in this case guppies (*Poecilia reticulata*).

In addition to the issue of environmental availability, limitation may occur post-consumption and be inherent in an individual's physiological need or ability to absorb and use carotenoids (Hill *et al*. 1994; Hudon 1994). Physiological demands are expected to vary in part because of an individual's genetic make-up. Despite the fact that genetic variance in a character is a crucial assumption in most evolutionary theories, heritabilities associated with carotenoid metabolism and colour expression have gone unexplored, with the exception of Hill's (1991) finding that the coloration of sons resembled fathers in house finches (*Carpodacus mexicanus*). Several investigators have found relationships that suggest carotenoids in birds may be compromised by challenges to an individual's health. Such results include associations between coloration, or carotenoid levels, and condition (Hill & Montgomerie 1994; Bortolotti *et al*. 1996), exposure to parasites (Bletner *et al*. 1966; Tyczkowski & Hamilton 1991) and environmental contamination (Eeva *et al*. 1998; Camplani *et al*. 1999).

While collectively these studies have confirmed a variety of potential mechanisms whereby carotenoids may

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become limited, their relative contribution within a population of animals is poorly understood. We provide a test of potential environmental, dietary, genetic and health components to explain variation in plasma carotenoids in wild American kestrels (*Falco sparverius*), a small, genetically monogamous (Villarroel *et al.* 1998) falcon.

The American kestrel has conspicuous, brightly coloured patches of bare skin at the lores (anterior to the eyes), the cere (above the bill) and the tarsi. They vary in colour from a dull yellow to a bright red-orange, which correlates with the quantity of carotenoids in their plasma (Bortolotti *et al.* 1996). As is typical of other sexually selected traits, males are the more brightly coloured sex, colours are most pronounced in the breeding season and young birds are duller than adults (Bortolotti *et al.* 1996; Negro *et al.* 1998). In addition to the availability of this background work on variation in carotenoids of captive birds, kestrels are a good model for investigating carotenoid availability given their diet. Many studies of carotenoid-based coloration have been conducted on granivores and frugivores (Hill *et al.* 1994; Gray 1996; Linville & Brietwisch 1997), which may have an abundance of carotenoids in their diet (Hudon 1994; Grether *et al.* 1999). As carnivores, kestrels are predicted to have difficulty in acquiring carotenoids (Gray 1996; Olson & Owens 1998; Hill 1999). In addition, these falcons have a broad diet including small mammals, birds, reptiles and insects (Bird 1988), and the relative abundance of primary prey species can be quantified on a per-territory basis (Bortolotti *et al.* 1991). As foods vary in carotenoid content (Goodwin 1984), we investigate potential associations between plasma concentrations of carotenoids and variation in abundance of the kestrel's major prey. Lastly, we performed a cross-fostering study to evaluate the relative importance of genetic and environmental sources of variation, including components of health, in determining plasma concentrations of carotenoids.

2. METHODS

(a) *Field methods*

We conducted this study in the boreal forest of north-central Saskatchewan, Canada (55° N, 106° W) where a population of around 150–200 pairs of American kestrels has been studied since 1988. Nesting chronology was determined accurately each year by repeated visits to all nest-boxes starting before laying and continuing until the eggs hatched (Tella *et al.* 2000*a*).

To obtain a blood sample for carotenoid analysis, we captured adult kestrels by hand in the nest-box during incubation, and nestlings were bled at 22 days of age just prior to their departure from the nest. About 0.5 ml of blood was extracted from the brachial or jugular vein. Samples were kept cool and plasma separated from the cellular components within a few hours and then frozen until carotenoid analysis.

In 1996 we conducted a preliminary study to examine the similarity of concentrations of carotenoids between members of mated pairs and between siblings (one male and one female per nest were selected randomly). We then performed a cross-fostering study in 1997 to partition the variance in carotenoid concentrations of nestlings into genetic and environmental components. We used four- and five-egg clutches (by far the most common clutches, Tella *et al.* 2000*a*) and manipulated them when the oldest chick was five days old, which assured us all chicks had hatched and avoided disturbance during the hatching span which could provoke stress (Wiebe & Bortolotti 1994; see also Tella *et al.* 2000*b*). We individually marked and weighed each nestling and ranked them by size. At each nest, we transferred two birds to a randomly chosen nest matched by hatching date and clutch size. The remaining nestlings were removed and transported in the same manner, and for the same length of time, as the translocated young, and then put back in their original nest to control for potential handling stress. All nests remained with the original number of young hatched, so that breeding chronology, breeding effort of adults and intranest competition of nestlings were held constant (Potti *et al.* 1999). Although we created broods where the same numbers of nestlings from two different families were raised together in the same nest, mortality resulted in unbalanced sample sizes. The final sample in our cross-fostering study was 172 birds in 44 nests.

We evaluated several variables representing the condition and health of nestlings when the blood sample was taken at 22 days of age. Kestrels were weighed and the tenth primary feather was measured (nearest mm) as an indicator of size. An index of body condition was calculated subsequently as the residuals from a regression of body mass on the cube length of the tenth primary ($r = 0.44$, $F_{1,140} = 15.15$, $p < 0.001$). We determined total plasma proteins in the field with a refractometer (Dawson & Bortolotti 1997). We also measured variables related to immune function. For leucocyte differentials (see §2(b)) we smeared a drop of blood on a microscope slide that was then air-dried and fixed in absolute ethanol. To evaluate T-cell-mediated immune response (CMI) we injected 50 µl of 1 mg ml^{-1} phytohaemagglutinin-P (Sigma, St Louis, MO, USA) in phosphate-buffered saline (PBS) subcutaneously in the right wing web. The left wing was injected with PBS as a control. The thickness of each wing web was measured with a micrometer (nearest 0.001 mm) just prior to and 24 h ($\pm$ 16 min) after injection. CMI was calculated as the difference in wing web swelling between right and left wings (Smits *et al.* 1999).

To establish variation in diets among nests, and hence variable exposure to carotenoids, we used video cameras installed near nest-boxes (Dawson 1999). We documented the number and sizes of prey items delivered to nestlings at 144 nests across 507 recording sessions for a total of 1370 h of observation between 1993 and 1995. We weighed samples of prey species in the field to estimate biomass delivered. To determine whether carotenoid concentrations of birds at a particular nest were associated with diet, we estimated the relative numbers of red-backed voles (*Clethrionomys gapperi*), the primary prey species, and other small mammals per territory. Snap traps were set on 54 and 34 territories in 1996 and 1997, respectively, during early July, a time corresponding to the end of incubation or early in the nestling period. An index of abundance (animals per 100 trap nights) was derived subsequently.

(b) *Laboratory analyses*

We used spectrophotometry to quantify total carotenoids (Tella *et al.* 1998). We diluted 0.1 ml of plasma with acetone (1 : 10), precipitated the flocculent protein by centrifugation and examined the supernatant in a Beckman DU 7400 spectrophotometer. We estimated concentration using a standard curve for lutein (alpha-carotene-3,3′-diol; Sigma), which is the predominant carotenoid as determined by high-performance liquid chromatography (P. Surai and G. R. Bortolotti, unpublished data).

For determination of leucocyte differentials, blood smears were stained with Wright–Giemsa stain, and the total white blood cell (WBC) count (cells $\times 10^9$ per litre) was estimated by taking the average number of leucocytes in five fields observed under $\times 40$ lens. WBC count was standardized to the number of red blood cells, and to control for variability in smear preparation the fields selected were in an evenly distributed monolayer. The proportions of heterophils, lymphocytes, monocytes, eosinophils and basophils were determined by identifying 100 cells under $\times 40$ power.

(c) *Statistical analyses*

Parent–offspring correlations were not possible in this study because adults could only be captured in numbers during incubation, and both food habits (G. R. Bortolotti and R. D. Dawson, personal observation) and carotenoids (Negro *et al.* 1998) vary seasonally. Instead, we calculated the intraclass correlation coefficient to estimate the fraction of total phenotypic variance attributable to factors causing resemblance between siblings. We obtained variance components by one-way ANOVA (Lessells & Boag 1987), and the standard error of the intraclass correlation coefficient following Becker (1984). Heritability (Falconer 1989) was estimated as twice the intraclass correlation coefficient of nestling measurements. These analyses were performed with SPSS (Norusis 1991).

Genetic and environmental effects on the concentrations of plasma carotenoids of offspring were estimated following a widely used approach (Merilä 1996; Potti *et al.* 1999). This consists of a two-factor nested ANOVA where the main effects were duplicate (a pair of nests matched by clutch size and hatching date) and nest of origin (nested within duplicates). The term duplicate accounts for any differences between pairs of nests. Within duplicates, the variation due to nest of origin estimates variation attributable to genetic transmission ($\frac{1}{2}V_A$), but also includes a quarter of the dominance variance ($\frac{1}{4}V_D$) and any pre-manipulation parental effects (V_P) if present. The term nest of rearing estimates the effects of the common environmental variance (V_{EC}), and error variance equals random environmental variation (V_{EI}) plus $\frac{1}{2}V_A + \frac{3}{4}V_D$. Analyses were performed with PROC GLM in SAS (SAS Institute 1988), using type III sums of squares due to unequal family sizes. Both duplicate and nest of origin were considered random effects, and therefore appropriate sums of squares and *F*-statistics were calculated using RANDOM in SAS.

We used the GLIM package (Crawley 1993) for generalized linear models (GLM) to determine which attributes of individual nestlings, or their rearing conditions, could account for variation in carotenoid concentrations. GLIM allowed us to simultaneously assess the contribution of a number of continuous and categorical explanatory variables, and their interactions, to the response variable, i.e. the concentration of carotenoids. Each explanatory variable was tested for significance in turn following the stepwise branching modelling procedure (Tella *et al.* 2000*a*). The result was the most adequate model for explaining variability in carotenoid concentration, where only significant explanatory variables were retained. For each fledgling, explanatory variables chosen were sampling date, laying date, clutch size, brood size, sex, body size (tenth primary length), mass, body condition, ranked hierarchy within brood at five days old, presence of mortality in the nest, total plasma proteins, white blood cell differentials and CMI. Since the response variable was normally distributed, we used the normal error and the identity link function for GLM modelling (Crawley 1993).

Table 1. *Results from a two-way nested ANOVA for concentrations of plasma carotenoids of fledglings in cross-fostered broods of American kestrels*

(Data were analysed in relation to rearing environment and family of origin nested within rearing. SS, sum of squares; MS, mean squares.)

source of variation	SS	d.f.	MS	*F*	*p*	r^2 (%)
origin	0.43	36	0.01	1.43	0.09	5.51
rearing	2.55	35	0.07	6.04	0.0001	61.35
residual	0.69	82	0.01			
model	3.16	71	0.04	5.20	0.0001	82.09

3. RESULTS

(a) *Genetic contribution*

The intra-class correlation coefficient for carotenoids in the preliminary study of 1996 indicated a high resemblance between full siblings in the same nest ($r = 0.49$, s.e. $= 0.23$, $F_{20,41} = 2.948$, $n_0 = 2$, $p = 0.009$). This sets an upper heritability of $h^2 = 0.987$ (s.e. $= 0.46$). These results emphasized the need for an experimental study to separate genetic from environmental components.

The two-factor nested ANOVA, performed on data from the cross-fostering study in 1997, showed that variation in plasma carotenoids was significantly explained by the nest where the bird was reared (61.35%), while the small variance explained by the nest of origin (5.51%) was not significant (table 1). The strong resemblance in carotenoids of siblings detected earlier was thus environmentally determined.

(b) *Attributes of individuals and local environments*

Despite the variety of characters used to describe attributes of individual nestlings and their rearing environments, the GLM model revealed that only a single variable, the proportion of lymphocytes, explained significantly ($F_{1,171} = 13.33$, $p < 0.001$) any variation in carotenoids (concentration of carotenoids $= 4.958 + 0.07998 \times$ proportion of lymphocytes). This model accounted for only 8.3% of the total original deviance. The proportion of heterophils was also significant in the first step of the statistical procedure, but significance disappeared when proportion of lymphocytes entered into the model. There were no significant interactions between the proportion of lymphocytes and other variables.

If the local effect of the nest was indeed important, as the nested ANOVA revealed, then we predicted that members of a mated pair should show similar carotenoid levels. Analysis of covariance (ANCOVA) with concentration of carotenoids in females as the dependent variable and year as a potential factor supported this prediction (concentration of carotenoids in males, $F_{1,97} = 7.85$, $p = 0.006$; year, $F_{1,97} = 0.64$, $p = 0.42$) (figure 1).

(c) *Dietary influence*

A total of 5195 prey items was observed, 5070 of which could be identified to some taxonomic level. The major components as a percentage by number (and biomass) of all prey delivered were 10.4 (47.6) small mammals, 63.5 (21.5) dragonflies (Odonata), 3.1 (12.8) birds, 6.4 (9.3)

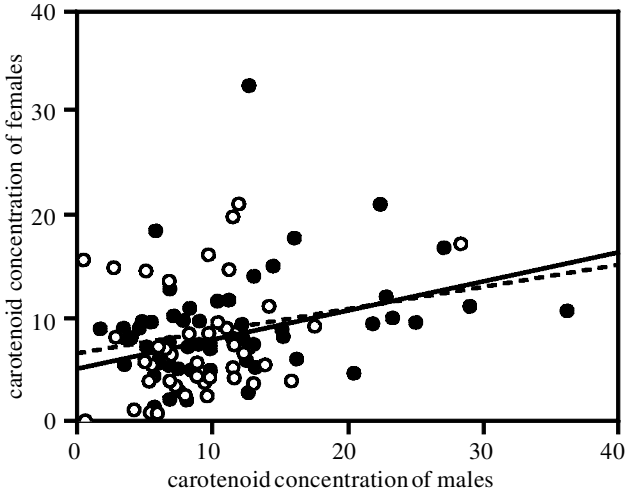

Figure 1. Scatter plot of concentrations of plasma carotenoids of male and female American kestrels in mated pairs during the incubation period in 1996 (solid circles and dashed line) and 1997 (open circles and solid line). There was no year effect.

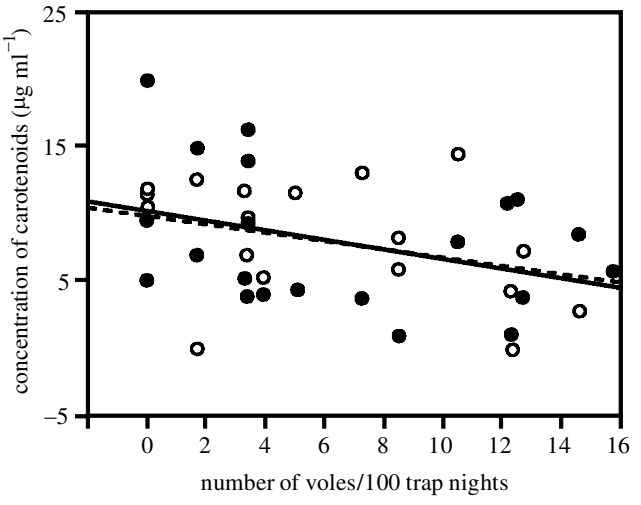

Figure 2. Scatter plot of concentrations of plasma carotenoids of male (open circles and line) and female (solid circles and dashed line) American kestrels during incubation against the number of voles found on their territories. There was no difference between the sexes.

frogs and 5.4 (1.8) grasshoppers (Orthoptera). Of primary interest here with respect to carotenoids is to establish that variation existed among nests. Vertebrates comprised, on average, 71.5% (s.e.m. = 1.89, $n = 144$) of the biomass delivered to each nest, but varied among nests from 0 to 100% (25% and 75% quartile values were 57.7% and 88.4%, respectively). The red-backed vole was the predominant prey species in numbers and biomass, with a mean of 39.3% (s.e.m. = 2.30, $n = 144$) of the biomass delivered to nests, but varied from 0 to 100% (25% and 75% quartiles were 18.7% and 61.9%, respectively).

Because the census lines for small mammals were randomly assigned to nest-boxes prior to the laying period, some areas were not used by kestrels or by pairs in the experiment. Therefore, we had sufficient sample sizes only for correlations between concentration of carotenoids of adult kestrels and prey abundance in 1996. Using adult carotenoid concentration as the dependent variable, ANCOVA was used to examine sex of adult as a factor and number of voles, laying date and date of blood sampling as potential covariates. Only the abundance of voles significantly explained carotenoid concentrations ($F_{1,37} = 5.59$, $p = 0.023$): the more prey on a territory, the lower the concentration of plasma carotenoids (figure 2). The analysis was repeated using total number of small mammals and similarly was significant ($F_{1,37} = 4.89$, $p = 0.033$).

4. DISCUSSION

Despite the importance of determining the heritability of traits, especially in evolutionary studies, this is the first study to present data on genetic versus environmental contributions to carotenoid concentrations. It is not clear whether additive genetic variance is in general more likely to be expressed under stressful or benign conditions (Hoffman & Parsons 1991), but heritabilities in wild birds have been found to be lower in poor environments (Gebhardt-Henrich & Van Noordwijk 1991; Merilä 1997; but see Merilä *et al.* 1999). The year we performed the cross-fostering experiment was average from the perspective of reproductive success and body mass of fledglings (ten-year data set, G. R. Bortolotti, unpublished data). Larger genetic variance may be expressed in very good years, but it is unlikely it would overcome the large environmental component that we found. Low heritability of a trait is usually related to its positive contribution to fitness, as strong directional selection may deplete genetic variation (Mousseau & Roff 1987; Roff 1997). Such an interpretation is consistent with the idea that carotenoids are important compounds for health or have advantages in mating. However, Merilä *et al.* (1999) provided evidence for weak directional selection for nestling condition, a fitness trait, allowing significant additive genetic variation to persist. The low heritability of fitness components does not necessarily mean that there is no additive genetic variation in these traits, but that this variation may be masked by higher environmental variances than in non-fitness traits (Merilä & Sheldon 1999).

Our multivariate search (using GLM) for non-dietary sources of environmental variation, i.e. rearing conditions, parental reproduction and attributes of individual nestlings, was largely unproductive. The exception being that leucocyte differentials explained significantly a small portion of variance. These findings are consistent with Saino *et al.*'s (1999) predictions that concentrations of plasma carotenoid should be correlated with 'variables reflecting the activation of the immune system,' (p. 445). Saino *et al.*'s (1999) study of barn swallows (*Hirundo rustica*) showed a correlation between plasma lutein and heterophils but not lymphocytes. Our GLM analysis revealed that lymphocytes were only marginally better than heterophils in explaining variation in plasma concentrations, but heterophils were dropped from the model when lymphocytes were included. What is important here at this early stage of investigation into immune function is to recognize that some association exists between parts of the immune system and carotenoids.

The large environmental component to variation in concentrations of carotenoids was best explained by differences among territories. The positive correlation between the concentrations of members of mated pairs (figure 1) suggests a local site-specific effect. It could also arise if kestrels paired assortatively with respect to colour or condition; the latter being demonstrated in this population by Bortolotti & Iko (1992). However, the adults examined here were bled over a month after pairing, and there may be pronounced seasonal changes in carotenoid concentrations (Negro *et al*. 1998). More plausibly the correlation between males and females is the result of a common diet, as females were fed by their mates (G. R. Bortolotti and R. D. Dawson, personal observation). This idea is further supported by the correlation between the abundance of voles and the levels of carotenoids in adults (figure 2). Those findings are consistent with studies of the great tit (*Parus major*) that have suggested carotenoid levels, or plumage colour, vary with the availability of lepidopteran larvae (Slagsvold & Lifjeld 1985; Eeva *et al*. 1998). Internest variation in diet is thus likely also to be responsible for the large environmental component found in our cross-fostering experiment (table 1).

As carotenoids vary among food types (Goodwin 1984), so do carotenoid levels and ultimately colour of consumers. We have shown that skin colour of adult American kestrels in captivity is in part a function of circulating levels of carotenoids (Bortolotti *et al*. 1996; Negro *et al*. 1998). Plasma concentrations of carotenoids and colour in these birds do vary even on a uniform diet, with some of the variance explained by condition (Bortolotti *et al*. 1996). When captive kestrels are maintained on a diet of laboratory mice, they lack almost all trace of skin colour and have plasma almost devoid of carotenoids (J. J. Negro, unpublished data).

The negative association between the abundance of voles and plasma carotenoids has important implications for the quality of the diet and perhaps ultimately the survival of the young. Studies of our kestrel population have shown that the abundance of voles has an impact on the growth, condition and survival of nestlings (Bortolotti *et al*. 1991; Wiebe & Bortolotti 1994, 1995). Therefore, the lower carotenoid levels in adult kestrels at nests with more voles suggest a potential trade-off between diet quality and quantity. While the role of food abundance in limiting reproduction of raptors has been well explored (Newton 1979), the potential for impacts of dietary micronutrients are unknown. For other birds, studies investigating prey selection based on nutrient quality other than energy content have shown variable results (Brodmann & Reyer 1999). Carotenoid concentrations found in nestlings in our study are also correlated with plasma levels of other nutrients, including vitamins A and E (P. Surai and G. R. Bortolotti, unpublished data). Recently Royle *et al*. (1999) proposed that variation in carotenoid levels in eggs within clutches of lesser black-backed gulls (*Larus fuscus*) may impact on nestling growth and survival. Similarly, immunocompetence and other health-related functions of may be influenced by carotenoids in eggs (Haq *et al*. 1996; Pastoret *et al*. 1998; Surai & Speake 1998; Tella *et al*. 2000*a*). Our results add to the growing concern over how conditions early in development may impact on animals later in life (Lindström 1999).

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