

## CAROTENOIDS AND SKIN COLORATION IN A SOCIAL RAPTOR

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**ABSTRACT.**—The outcome of social and sexual competition in animals is typically mediated through the expression of body traits. Conspicuous characters such as yellow, orange, and red colorations in skin, scales, and feathers are often posited as quality-dependent signals, because such colors are made of dietary carotenoids and their use for signaling conflicts with health functions. Raptors often lack brightly colored feathers but most diurnal species display intense orange and yellow hues in the cere and legs. Here we test the hypothesis that integument coloration functions as a signal of status in wild raptors, revealing availability of carotenoid pigments. As study model we used the Black Kite (*Milvus migrans*), a highly social, long-lived, and sexually monogamous Accipitiform. Regular trapping of adults throughout the breeding season revealed that circulating carotenoid levels were highest in breeding males, whereas breeding females and floaters showed moderate and statistically similar carotenoid titers. Plasma levels of carotenoids showed a slight seasonal decline, especially marked in post-laying females. Leg and cere colorations were visually assessed through comparison to a color chart, yielding a very high inter-observer reliability and consistency with simultaneous spectrophotometric measures. Integument color was similar between the sexes, brighter in breeders compared to floaters, and positively related to circulating carotenoids, but only in the floaters. These results suggest that the physiological regulation of signal expression is different in breeders and floaters, possibly involving a costly social or physiological mechanism that ensures the honesty of coloration as a quality-dependent signal. In addition, breeding males and females displayed similar color scores despite strong differences in plasma carotenoids, indicating sex-related differences in physiological regulation that were not apparent in floaters. Our results are consistent with a role of integument coloration in status signaling in wider competitive contexts than those enforced by sexual selection. The reported patterns of coloration can be ultimately explained by two alternative, non-mutually exclusive hypotheses: (1) the lower coloration displayed by floaters reflects underlying physiological limitations and mediates the access to breeding resources through social competition (constraint hypothesis), and (2) young floaters have evolved mechanisms to restrain color expression and thus signal their competitive inferiority, avoiding physiological and social costs (restraint hypothesis).

**KEY WORDS:** *Black Kite; Milvus migrans; carotenoids; coloration; constraint; dominance; restraint; sexual selection; status signaling.*

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### CAROTENOIDES Y COLORACIÓN DE LA PIEL EN UNA RAPAZ SOCIAL

**RESUMEN.**—El resultado de la competencia social y sexual en animales es a menudo facilitado por la expresión de caracteres corporales. Las tonalidades amarillas, anaranjadas y rojas que colorean la piel, escamas o plumas de los vertebrados suelen funcionar como señales indicadoras de la calidad del individuo, pues dependen de la ingesta de pigmentos carotenoides y su utilización ornamental impide otros usos relacionados con la salud. Las aves rapaces suelen carecer de plumajes vivamente coloreados, pero la

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mayoría de las especies diurnas muestran intensas tonalidades amarillas y anaranjadas en la piel de la cera y las patas. En el presente estudio comprobamos la hipótesis de que la coloración del tegumento funciona como una señal de estatus social en rapaces silvestres, revelando la disponibilidad de pigmentos carotenoides. Como modelo de estudio utilizamos a *Milvus migrans*, un Accipitriforme altamente social, longevo y monógamo. La captura y muestreo regular de adultos a lo largo de la estación de cría mostró niveles máximos de carotenoides circulantes en los machos reproductores, mientras que las hembras y los individuos flotantes tuvieron niveles moderados y estadísticamente similares. Las concentraciones plasmáticas de carotenos sufrieron una ligera disminución estacional, especialmente marcado en las hembras reproductoras tras la época de puesta. La coloración de las patas y la cera fue estimada visualmente por comparación con una tabla de colores, mostrando una elevada repetición entre observadores y siendo consistente con mediciones espectrofotométricas realizadas en los mismos sujetos. La coloración del tegumento fue similar en machos y hembras, fue más elevada en aves reproductoras que en flotantes, y estuvo positivamente asociada a los niveles de carotenoides plasmáticos de aves flotantes. Nuestros resultados sugieren que la regulación fisiológica de la coloración externa es diferente en aves reproductoras y flotantes, y posiblemente implica mecanismos costosos de naturaleza social o fisiológica que aseguran el mantenimiento del color como una señal honesta de calidad. Además, los machos y las hembras reproductivas mostraron coloraciones similares a pesar de diferir significativamente en los niveles de carotenoides circulantes, evidenciando un dimorfismo sexual en la regulación fisiológica que no fue aparente en el grupo de aves flotantes. Nuestros resultados son consistentes con un papel de la coloración tegumentaria en la señalización del estatus en contextos competitivos amplios, no exclusivamente enfocados a los procesos de selección sexual. Los patrones de coloración aquí mostrados pueden ser en último término explicados por dos hipótesis alternativas y no excluyentes, a saber: (1) la menor intensidad de coloración de los flotantes manifiesta limitaciones fisiológicas que impiden su acceso a los recursos reproductivos mediante competencia (hipótesis de "constricción"), y (2) los jóvenes flotantes han desarrollado adaptaciones para restringir su expresión de color y así mostrar su inferioridad competitiva, evitando costos fisiológicos y/o sociales que aseguran la honestidad de estas señales (hipótesis de "restricción").

[Traducción del equipo editorial]

The evolution of brightly colored traits in animals has attracted biologists since Darwin, who proposed their role as ornaments involved in mate choice and intrasexual competition (Darwin 1871, Anderson 1994). In birds, the widespread presence of red, orange, and yellow-colored feathers and bare skin patches depends on carotenoids (Brush 1990, McGraw and Hill 2006), a family of pigments produced by plants, algae, and fungi that animals can only obtain through diet (Armstrong and Hearst 1996). Reduced access to dietary carotenoids results in a loss of coloration (Brush 1981) and carotenoid-rich food enhances color expression (Casagrande et al. 2007, Sternalski et al. 2012). In addition to carotenoid intake, birds may differ in circulating carotenoid levels and integument color when fed a constant diet, indicating that endogenous (physiological) regulation takes place after ingestion and is affected by factors such as age, sex, season, hormone levels, health status, and body condition (Bortolotti et al. 1996, 2000, Negro et al. 1998, Blas et al. 2006). Because carotenoids are also important micronutrients and have antioxidant and immunostimulant effects (Møller et al. 2000, Chew and Parks 2004), their allocation to color ornamentation

conflicts with their use for health functions, generating a trade-off that ensures the honesty of bright coloration as a condition-dependent trait: only superior-quality individuals can afford the brightest colorations without compromising their health (Blount et al. 2003, Faivre et al. 2003, Alonso-Alvarez et al. 2004, Dawson and Bortolotti 2006, Martínez-Padilla et al. 2007).

Diurnal birds of prey typically show brightly colored skin in the legs and around the bill, which often vary according to sex and age (e.g., Sarasola et al. 2011, McDonald 2003). The live nature of these soft tissues implies, in contrast to feathers, that coloration can change rapidly if carotenoid constituents are diverted to health functions (Faivre et al. 2003, Perez-Rodriguez 2008), allowing a dynamic and more accurate indication of the current condition of the individual. Explicit evidence that carotenoid-based coloration reflects individual quality in raptors comes from studies on small-sized species displaying a striking sexual dimorphism, namely kestrels and harriers. Skin color in adult males correlates with body size, nutritional condition, feather growth rate, and territory quality in American Kestrels (*Falco sparverius*; Bortolotti et al. 2003,

Bostrom and Ritchison 2006), with hunting efficiency, territory quality, and onset of breeding in Eurasian Kestrels (*Falco tinnunculus*; Casagrande et al. 2006, Vergara and Fargallo 2011) and with body condition in Montagu's Harriers (*Circus pygargus*; Mougeot and Arroyo 2006). Breeding females, young birds, and also males outside the reproductive season typically show lower circulating carotenoids and skin coloration, suggesting a major role of carotenoid-based traits in sexual selection (e.g., Bortolotti et al. 1996, Negro et al. 1998, Casagrande 2011). Because kestrels and harriers show some of the more striking levels of sexual color dimorphism in raptors, our understanding of carotenoid-based signals is largely biased toward evolutionary scenarios characterized by strong sexual selection (Anderson 1994), precluding generalization of findings and conclusions to sexually monomorphic raptors (but see Sarasola et al. 2011 for Chimango Caracara [*Milvago chimango*]) and broader social contexts. To fill this gap, we tested the hypothesis that bright coloration is a signal of status in the Black Kite (*Milvus migrans*), a long-lived, monogamous, and highly social raptor. Contrary to kestrels and harriers, male and female Black Kites look alike and, except for the larger mass of females during breeding, body size overlaps to a large extent between the sexes (Sergio et al. 2007a). In addition, Black Kites display long-term pair bonds (Forero et al. 1999), suggesting a lower intensity of sexual competition compared to kestrels and harriers, which frequently change mates between breeding seasons (Village 1985, Mougeot et al. 2001, Steenhof and Peterson 2009). As in most long-lived species, Black Kites show deferred reproduction and first breeding usually occurs when birds are 2–7 yr old (Blas et al. 2009). Consequently, wild populations are typically composed of breeding birds coexisting with non-territorial floaters, which often forage and roost communally (Sergio et al. 2009a). In the study population, social confrontations are frequently observed and involve competition for accessing mates and breeding territories, and also disputes over food resources (Sergio et al. 2007b, 2009a, 2011a). If Black Kites have evolved carotenoid-based color traits to signal their social status, we may expect breeders to display brighter colorations compared to floaters. If carotenoid-based signals have a major role in sexual selection, we may expect males to display brighter colors compared to females, and maximum levels of signal expression during the fertile period of the breeding season (Negro et al. 1998).

#### METHODS

**Field Procedures and Blood Sampling.** Black Kites ( $n = 127$ ) were captured using cannon nets throughout the breeding season (from early March to late July) in Doñana National Park (southwestern Spain). The study population has been the subject of long-term scientific monitoring (since 1965), and since 1986 birds have been regularly banded with alphanumerically-coded rings, allowing individual identification of the captured birds with the use of spotting scopes (e.g., Forero et al. 1999, Blas et al. 2009, Sergio et al. 2011a, 2011b). Intensive field surveys allowed us to identify breeders ( $n = 77$ ) when trapped individuals were found holding a breeding territory. Floaters do not defend exclusive home ranges and are often gregarious, foraging in undefended communal areas, and gathering at night at several communal roosts. Monitoring of the communal roosts twice a week allowed identification of floaters ( $n = 50$ ; details in Sergio et al. 2009a).

After the capture of each kite, we collected a 1-ml blood sample from the brachial vein, transferred it to a heparinized tube, and stored it in a cooler until centrifugation later on the same day. Plasma was stored frozen ( $-80^{\circ}\text{C}$ ) until quantification of carotenoid levels, and the cellular fractions were preserved in ethanol for molecular sexing (see below). The collected samples were assigned to two discrete seasonal stages: pre-laying and post-laying. Blood samples collected at the beginning of the breeding season (March and April) were assigned to the pre-laying stage, which corresponds to spring arrival, territory acquisition, and mating (Blas et al. 2010). This period is characterized by elevated levels of sex steroid hormones (testosterone in males and estradiol in females, Blas et al. 2010, Blas and Hiraldo 2010) allowing comparisons between breeders and floaters during the fertile time of the year, when the reproductive endocrine axis is typically upregulated (Blas et al. 2011). Samples collected during May and June were assigned to the post-laying stage, characterized by lower levels of circulating testosterone and estradiol (see Blas and Hiraldo 2010 for details).

**Color Quantification and Validation Study.** The colors of unfeathered ceres and tarsi were scored by comparing the bird under shaded light conditions with a color chart derived from paint samples, a method previously used in numerous studies of avian coloration (e.g., Bortolotti et al. 1996, Bostrom and Ritchison 1996, Negro et al. 1998, Figuerola et al. 1999, Sternalski et al. 2010, 2012). In

particular, we used the exact same color chart previously utilized in studies assessing carotenoid-based coloration in the cere, lore, and legs of American Kestrels (Bortolotti et al. 1996, Negro et al. 1998). However, we discarded the two brighter, red-orange options as they were outside the natural range of variation in Black Kites. We also reversed the numeric order of the color options, allowing higher numbers to represent brighter colors (as opposed to the studies above) and presumably higher carotenoid content. Therefore cere and tarsi were each scored from 1 (dull yellow) to 4 (bright yellow-orange). Direct comparison of our color chart to standard Munsell color chips resulted in the following correspondence for Hue, Value (or "Lightness") and Chroma, the latter two shown within brackets: score 1 = 5 Yellow (9/6); score 2 = 5 Yellow (8.5/10); score 3 = 2.5 Yellow (8/12) and score 4 = 8.75 Yellow-Red (7/14). As the color score increases from 1 to 4, the hues of the color chips increase towards orange and away from yellow (possibly indicating increased carotenoid pigment deposition), with increasing chroma scores indicating a more pure color (i.e., decreasing in brightness; Munsell 1912). In order to test the reliability of color classification by different observers using the same color chart, the leg colorations of 18 trapped adult kites were simultaneously assessed by two independent raters (AT and LL), and an additional independent sample of seven birds was simultaneously assessed by three independent observers (AT, FS, and a field assistant). Raters coincided in assigning the same color score to kites in 91.5% of the occasions. The inter-observer reliability coefficient was  $Kappa = 0.86$  ( $P < 0.01$ ), which is interpreted as an "almost perfect" agreement (Landis and Koch 1977). Although spectrophotometric assessment of coloration was impractical for all kites because of logistical and funding constraints, we were able to estimate the reliability of the chart method on 25 trapped adult kites which were simultaneously assessed by the color chart and by spectral measures of leg coloration, using a Minolta CM-2500c spectrophotometer (Minolta, Osaka, Japan) operating at wavelengths 360–700 nm.

The spectral pattern of the leg integuments (Fig. 1A) showed minimum reflectance values around 450 nm and a marked increase from 500 nm onwards, reaching maximum levels in the interval 620–700 nm. Given this pattern, for each of the 25 kites spectra we calculated total brightness by summing all percentage reflectance values in the

region 360–700 nm. We then calculated two color variables: (a) "carotenoid saturation" *sensu* Montgomerie (see page 113 in Montgomerie 2006), as the ratio between the total reflectance in the region of interest (the interval 550–700 corresponding to yellow, orange, and red wavelengths) and total brightness, and (b) "carotenoid chroma" *sensu* Andersson and Prager (see page 78 in Andersson and Prager 2006), as the difference in reflectance between 700 nm and 450 nm divided by the reflectance at 700 nm. Visual classification was consistent with the color values obtained through spectrophotometry: color score was positively associated with carotenoid saturation *sensu* Montgomerie 2006 ( $R_s = 0.64$ ,  $P < 0.001$ ,  $n = 25$ ) and with carotenoid chroma *sensu* Andersson and Prager 2006 ( $R_s = 0.61$ ,  $P < 0.001$ ,  $n = 25$ ). Furthermore, birds classified as score = 1, 2 and 3 showed significant differences in the spectrophotometric-derived color variables (carotenoid saturation  $F_{2,25} = 7.21$ ,  $P < 0.01$ ; carotenoid chroma  $F_{2,25} = 6.60$ ,  $P < 0.01$ ; see Figs. 1B–1C). Thus, a visually higher color score was truly indicative of a more yellow-orange and more saturated color. Finally, because we were interested in the overall carotenoid-based skin coloration (and the overall amount of pigments used), we summed cere and tarsi scores (which were positively correlated,  $R_s = 0.61$ ,  $P < 0.001$ ,  $n = 127$ ) to obtain a single total skin color value per individual, conforming to previous studies (e.g., Bortolotti et al. 1996, Bostrom and Ritchison 1996, Negro et al. 1998, Sternalski et al. 2011, 2012).

**Laboratory Analyses.** Total plasma carotenoid levels were quantified following Bortolotti et al. (2000). Plasma samples were diluted with acetone and the flocculant proteins were separated through centrifugation at 1500 g for 10 min. The optical density of the carotenoid peak at 476 nm was determined in the supernatant, and plasma levels were estimated by interpolation using a standard curve of lutein (alpha-carotene-3, 3'-diol; Sigma-Aldrich), the predominant plasma carotenoid in all the raptor species analyzed so far (e.g., Negro et al. 2002, Laaksonen et al. 2008, Sternalski and Mougeot 2010). Sex determination was performed through molecular analyses using the cellular fractions of the blood samples preserved in ethanol. Following Ellegren (1996), we performed a PCR multiplex amplification of DNA fragments located in the CHD gen using the primers 3224R (E1), cfr (E2) and 2945F (E3).

**Statistical Analyses.** We analyzed data using Generalized Linear Models GLMs (McCullagh and

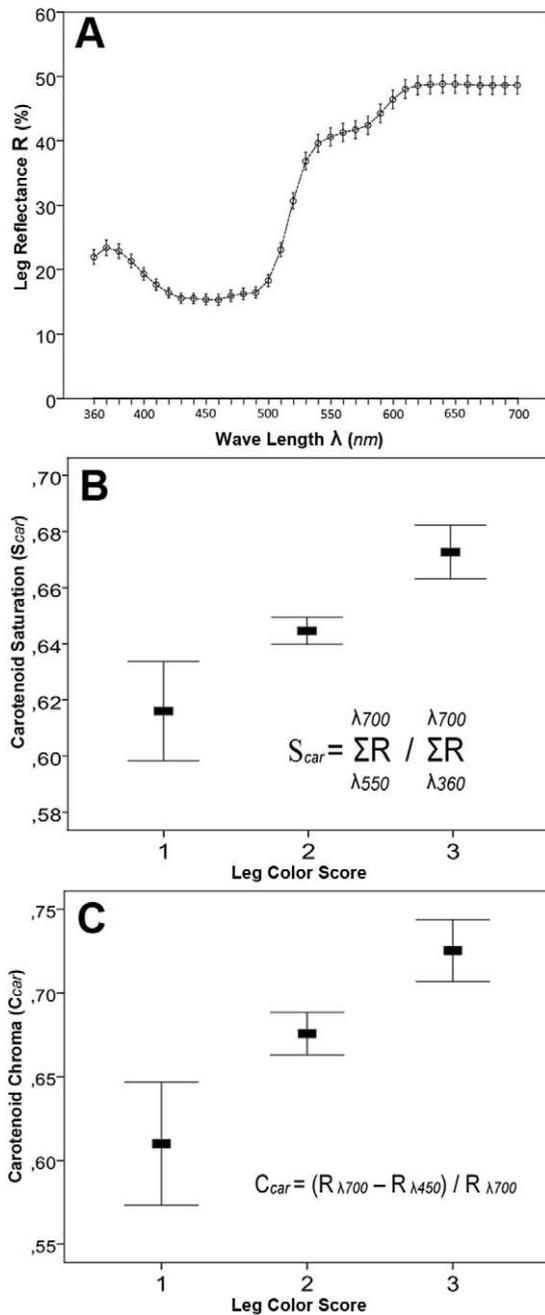


Figure 1. (A) Reflectance pattern of the integument of Black Kites' legs (average  $\pm$ SE values across 35 intervals of 10 nm;  $n = 25$  birds) in the spectrum range 360–700 nm according to a Minolta CM-2500c spectrophotometer. (B) Average ( $\pm$ 1 SE) levels of carotenoid saturation *sensu* Montgomerie 2006 (ratio between the total reflectance in the interval 550–700 nm and total brightness) in Black

Nelder 1989). Carotenoid concentration was not normally distributed but normality was achieved by log-transformations. Our GLMs testing individual variation in plasma carotenoids included sex, status and season as potential explanatory variables. A second GLM using color scores as dependent variable and sex, status, season, and plasma carotenoids was also fitted. Explanatory variables in the initial saturated GLMs also included the two-way interactions between main factors. All GLMs were modeled using a normal distribution of errors and identity link function, and following a backwards stepwise procedure where the least significant terms were sequentially removed, starting with the two-way interactions, until obtaining a minimum adequate model that only retained significant terms with  $P \leq 0.05$ , following Zuur et al. (2007).

RESULTS

Circulating carotenoid concentration showed an overall seasonal decline between pre- and post-laying, was similar in males and females, and higher in breeding birds compared to floaters (Table 1). However, the latter two results were driven by the group of breeding males displaying higher carotenoid concentrations compared to all the other birds combined (Fig. 2; *post-hoc* tests  $P < 0.05$  for all the comparisons between breeding males and any other sex-status combination and  $P > 0.20$  for all the other comparisons). None of the other two way interactions were significant (Table 1).

Integument coloration showed an overall weak but highly significant association with plasma carotenoids (Fig. 3;  $R_s = 0.23$ ,  $P < 0.01$ ). The GLM revealed that coloration was systematically higher in breeders compared to floaters (Table 2), and was similar during pre- and post-laying (Fig. 4). Within each group of status, males and females displayed similar color scores (Table 2). The significant interaction Status x Carotenoids indicated that color was positively related to circulating carotenoid concentrations in floaters ( $\chi^2 = 12.43$ ,  $df = 1$ ,  $P < 0.01$ ).

←

Kites' legs as a function of color scoring performed by visual comparison to a color chart ( $n = 25$  birds). (C) Average ( $\pm$  1 SE) levels of carotenoid chroma *sensu* Andersson and Prager 2006 (difference in reflectance between 700 nm and 450 nm divided by the reflectance at 700 nm) in Black Kites' legs as a function of color scoring performed by visual comparison to a color chart ( $n = 25$  birds).

Table 1. Summary of results from the GLM explaining plasma carotenoid concentration in Black Kites ( $n = 127$  birds). The estimated effect and standard error as well as  $\chi^2$  and associated probabilities are shown for those variables that significantly improved the fit of the model. For those terms that were excluded from the model during the backwards procedure,  $\chi^2$  and  $P$  values when added to the final model are given.

EFFECT	ESTIMATE $\pm$ SE	DF	$\chi^2$	$P$
Intercept	0.61 $\pm$ 0.05			
Sex	-0.08 $\pm$ 0.06	1	1.28	0.26
Status	-0.04 $\pm$ 0.06	1	3.92	0.05
Study period	0.08 $\pm$ 0.04	1	3.70	0.05
Sex X Status	0.24 $\pm$ 0.08	1	8.84	<0.01
Sex X Study period	—	1	0.12	0.73
Status X Study period	—	1	0.04	0.84

Fig. 3) but not in breeding birds ( $\chi^2 = 0.05$ ,  $df = 1$ ,  $P = 0.83$ ). None of the other factors or two-way interactions were statistically significant (Table 2).

#### DISCUSSION

**Social Function of Skin Color.** Our study supports the idea that carotenoid-dependent coloration in Black Kites is a social signal of status, likely associated with individual quality. Breeding kites are generally older and socially dominant during competition with floaters for breeding and feeding resources (Sergio et al. 2009a, 2011a), and age *per se* implies improved survival and reproductive capabilities and thus higher quality (Blas et al. 2009, Sergio et al. 2009b, 2011b). The expression of brightly colored

legs and ceres in breeding kites may thus have evolved as a mechanism to convey information of their competitive superiority, allowing conflict resolution while avoiding injuries and physical fights. Although there is ample evidence that carotenoid-dependent traits function as a signal of status in a range of avian species, intraspecific variability in the skin color of adult raptors has been largely interpreted in a sexual selection context (Bortolotti 1996, Negro 1998, Mougeot and Arroyo 2006, Casagrande et al. 2007, 2011, Sarasola et al. 2011). Dominance-related traits in Black Kites are likely relevant in mate choice and intrasexual competition. However, skin coloration was similar between breeding

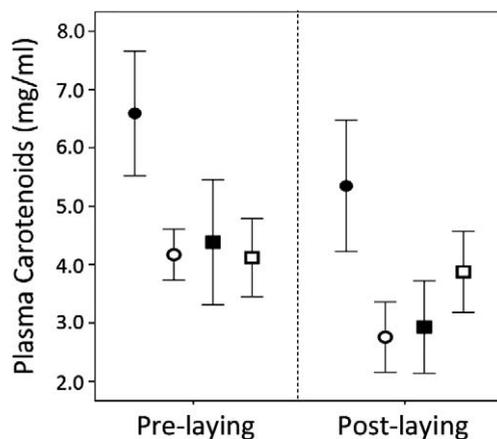


Figure 2. Mean  $\pm$ SE circulating levels of carotenoids in relation to sex and status of Black Kites during the pre- and post-laying stages of the breeding cycle. Breeders and floaters are represented by circles and squares, respectively. Males and females are represented by filled and open symbols, respectively.

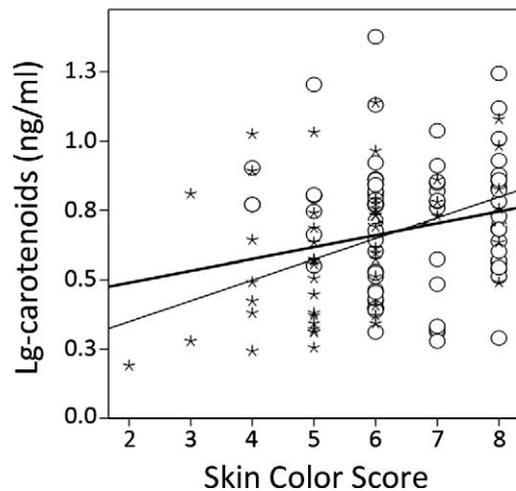


Figure 3. Total skin color score (sum of cere and leg scores, estimated by comparison to a color chart) in Black Kites, as a function of circulating levels of carotenoids (log-carotenoids ng/ml) in breeders (circles) and floaters (asterisks). Regression lines are shown for floaters (thin line) and all birds cumulated (thick line).

Table 2. Summary of results from the GLM explaining integument coloration in Black Kites ( $n = 127$  birds). The estimated effect and standard error as well as  $\chi^2$  and associated probabilities are shown for those variables that significantly improved the fit of the model. For those terms that were excluded from the model during the backwards procedure,  $\chi^2$  and  $P$ -values when added to the final model are given.

EFFECT	ESTIMATE $\pm$ SE	DF	$\chi^2$	$P$
Intercept	4.03 $\pm$ 0.45			
Sex	—	1	0.02	0.88
Status	2.68 $\pm$ 0.62	1	17.58	<0.01
Study period	—	1	0.13	0.71
Carotenoids	2.40 $\pm$ 0.68	1	6.39	0.01
Sex X Status	—	2	0.95	0.62
Sex X Study period	—	3	0.18	0.98
Sex X Carotenoids	—	1	0.02	0.90
Status X Study period	—	2	0.22	0.90
Status X Carotenoids	-2.52 $\pm$ 0.89	1	7.80	<0.01
Study period X Carotenoids	—	1	0.73	0.39

males and females and remained unchanged throughout the breeding season. The lack of sexual differences and the lack of a seasonal peak during pre-laying suggest that communication through leg and cere color signals apply to broader competitive contexts. As opposed to previous studies, we were also able to assess carotenoid and coloration in a sample of floaters, a social group that generally makes up a considerable fraction of avian populations but is typically hard to sample (Zack and

Stutchbury 1992, Newton and Rothery 2001, Blas and Hiraldo 2010, Penteriani et al. 2011). As it occurred in breeders, the simultaneous lack of sexual and seasonal variation in floaters color further supports the idea that the bright integument signals year-round individual quality, which would be particularly adaptive in highly social raptors like Black Kites, which may forage, migrate and roost communally in both the breeding and wintering quarters (Cramp and Simmons 1980, Del Hoyo et al. 1994). Based on these results, we recommend a broader appreciation of skin color function, which may not be solely associated with sexual selection. Similarly, recent research in raptor nestlings indicates that dominant siblings within a brood display brighter colorations (Sternalski et al. 2012), suggesting that carotenoids may be preferentially allocated to skin coloration under increased competition and highly social environments, as it seems to be the case in our saturated population (Sergio et al. 2009a, 2011a).

**Proximate Mechanisms of Color Expression.** Breeders and floaters seemed to differ in how they allocated circulating carotenoids to develop external colorations. Although an overall highly significant association between circulating carotenoids and skin color provides supporting evidence that the bright integument originates from carotenoid pigments, the correlation was absent in breeding birds, which displayed the highest color scores. In addition, breeding males and females showed strong differences in plasma carotenoid levels despite similar cere and leg color scores. Altogether,

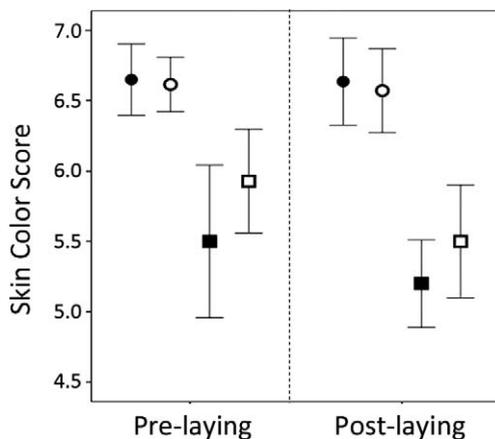


Figure 4. Mean  $\pm$ SE total skin color score (sum of cere and leg scores, estimated by comparison to a color chart) in relation to sex and status of Black Kites during the pre- and post-laying stages of the breeding cycle. Breeders and floaters are represented by circles and squares, respectively. Males and females are represented by filled and open symbols, respectively.

these results suggest that the proximate mechanisms allowing carotenoid incorporation into the showy integument are more complex than previously appreciated and highly sex- and status-dependent. If floaters were immunosuppressed or suffered oxidative stress, their demand for plasma carotenoids would jeopardize skin color development (Faivre et al. 2003, Constantini et al. 2010), explaining the reported patterns. However, the positive correlation between plasma carotenoids and skin color strongly suggests that ornament expression was a major allocation priority for nonbreeders, as would be expected in healthy and immunocompetent individuals. On the contrary, the plasma-skin color association was not significant in breeding birds, suggesting that they were delivering more carotenoids to functions other than integument ornamentation (i.e., health, egg formation) compared to floaters, without compromising signal development. Reproduction requires a considerable extra energy investment involving physiological costs such as increased blood parasites and oxidative stress, and reduced immune function (Wiersma et al. 2004, Knowles et al. 2009, Casagrande et al. 2011). In males, elevated testosterone may facilitate carotenoid absorption (Blas et al. 2006), partially explaining the reported higher plasma levels in breeding males (but see Blas and Hiraldo 2010), although it also generates immunosuppression and oxidative damage (Folstad and Karter 1992, Alonso-Alvarez et al. 2007), which should affect the trade-off in carotenoid allocation to signaling. In females, egg formation demands large amounts of carotenoids (Blount et al. 2000), possibly explaining why breeders tended to decrease circulating levels after laying. However, the potential cost of reproduction did not compromise ornament expression, and skin color remained above the level of floaters throughout the breeding season. The precise physiological mechanisms explaining carotenoid allocation patterns in breeders and floaters remains to be elucidated, but our results strongly suggested that breeders are more efficient at incorporating carotenoids from the blood stream to the colored integument. Such status-dependent efficiency in ornament development and the dissociation between plasma carotenoid levels and external coloration suggests that breeding kites are coping with increased carotenoid demands for health and reproductive functions while maintaining the highest levels of ornament expression, which supports the idea that carotenoid-dependent leg and cere colorations are honest signals of individual quality.

**Ultimate Factors Allowing Honesty.** The evolution of traits indicative of individual quality relies on the existence of heavy costs associated with signal development, expression, and maintenance. Such costs are expected to be only affordable by superior phenotypes, precluding “cheating” by low-quality individuals and enforcing signal honesty and stability (Zahavi 1975; for reviews see Maynard Smith and Harper 2003, Bradbury and Vehrencamp 2011). Research on the honesty of bright ornaments as quality signals is largely biased toward the understanding of physiological costs, because carotenoids possess antioxidant and immune-stimulant properties (Møller et al. 2000; Chew and Parks 2004) and allocation to color ornaments potentially compromises health functions (Blount et al. 2003, Faivre et al. 2003, Alonso-Alvarez et al. 2004, Dawson and Bortolotti 2006, Martínez-Padilla et al. 2007). However, not all carotenoids provide the same health benefits (Constantini and Moller 2008) and alternative explanations ensuring the honesty of carotenoid-dependent ornamentation in raptors have been largely overlooked. Even if the proximate (physiological) mechanisms allowing color development were “inexpensive,” the stability of color signaling could be promoted by social interactions, because the avoidance of brightly colored, dangerous fighters should save time, energy, and potential injuries to both dominants and subordinates (e.g., Studd and Robertson 1985, Mateos and Carranza 1997). A recent study provides experimental evidence for social mechanisms enforcing the honesty of quality traits in Black Kites. Breeding kites from our population decorate their nests exuberantly with human-made objects (mostly white plastic), which act as a threat to conspecifics by revealing the overall quality of the signaler (i.e., its territory quality, age, viability, and fighting ability; Sergio et al. 2011a). Lower-quality individuals abstain from signaling either by refraining from collecting experimental items offered near their nest or by removing experimental items placed in their nest. Experimentally augmented decorations increase the rate of social challenges, revealing a social cost of signal expression: only higher-quality individuals are capable of meeting such costs and these birds elevate their attack rate on intruders to match the experimentally augmented decoration-level (Sergio et al. 2011a).

**Conclusions.** Our study supports the idea that the bright colorations of the legs and ceres of Black Kites function as a signal of status, revealing the allocation of circulating carotenoids to ornamentation and possibly indicating individual quality. The lower level of signal expression displayed by floaters

can be explained by two alternative hypotheses: (i) they are functionally unable to incorporate more circulating pigments into the showy integument due to physiological constraints, or (ii) they are physiologically capable of increasing color development, but it is beneficial to them to restrain or delay ornament expression to avoid social and health-related costs. Although the constraint and the restraint hypotheses were originally proposed to explain the lower reproductive performance of young birds (Curio 1983, Desrochers 1992, Blas and Hiraldo 2010), they could as well apply to age-related changes in ornament expression leading to color differences between floaters and breeders. In fact, both mechanisms could operate simultaneously: if individual quality increases with age, the allocation of carotenoids to ornamentation will incur progressively lower costs as birds age, reducing the adaptive value of restraining color expression. As suggested for the evolution of delayed plumage maturation, status signaling through paler skin color in floaters could be a strategy to maximize fitness in circumstances where young adults cannot effectively compete with older birds for breeding resources (Gerard et al. 2012). Such explanation would be fully consistent with the endocrine profiles that characterize our Black Kite population, where young floating birds appear to be sexually competent to initiate breeding but highly susceptible to physiological stress imposed by dominant breeders (Blas et al. 2011), which despotically monopolize a limited number of suitable breeding territories (Sergio et al. 2007b, 2009a).

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## LITERATURE CITED

- ALONSO-ÁLVAREZ, C., S. BERTRAND, G. DEVEVEY, M. GAILLARD, J. PROSA, B. FAIVRE, AND G. SORCI. 2004. An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. *American Naturalist* 164:651–659.
- , ———, B. FAIVRE, O. CHASTEL, AND G. SORCI. 2007. Testosterone and oxidative stress: the oxidation handicap hypothesis. *Proceedings of the Royal Society of London Series B* 274:819–825.
- ANDERSSON, M. 1994. Sexual selection. Princeton Univ. Press, Princeton, NJ U.S.A.
- ANDERSSON, S. AND M. PRAGER. 2006. Quantifying colors. Pages 78 in G.E. Hill and K.J. McGraw [Eds.], Bird coloration, Vol. 2: function and evolution. Harvard Univ. Press, Cambridge, MA U.S.A.
- ARMSTRONG, G.A. AND J.E. HEARST. 1996. Carotenoids 2: Genetics and molecular biology of carotenoid pigment biosynthesis. *FASEB Journal* 10:228–237.
- BLAS, J. AND F. HIRALDO. 2010. Proximate and ultimate factors explaining floating behavior in long-lived birds. *Hormones and Behavior* 57:169–176.
- , L. LÓPEZ, A. TANFERNA, F. SERGIO, AND F. HIRALDO. 2010. Reproductive endocrinology of wild, long-lived raptors. *General and Comparative Endocrinology* 168:22–28.
- , L. PÉREZ-RODRIGUEZ, G.R. BORTOLOTTI, J. VIÑUELA, AND T.A. MARCHANT. 2006. Testosterone increases bioavailability of carotenoids: insights into the honesty of sexual signalling. *Proceedings of the National Academy of Science USA* 103:18633–18637.
- , F. SERGIO, AND F. HIRALDO. 2009. Age-related improvement in reproductive performance in a long-lived raptor: a cross-sectional and longitudinal study. *Ecography* 32:647–657.
- , ———, J.C. WINGFIELD, AND F. HIRALDO. 2011. Experimental tests of endocrine function in breeding and nonbreeding raptors. *Physiological and Biochemical Zoology* 84:406–416.
- BLOUNT, J.D., D.C. HOUSTON, AND A.P. MØLLER. 2000. Why egg yolk is yellow. *Trends in Ecology and Evolution* 15:47–49.
- , N.B. METCALFE, T.R. BIRKHEAD, AND P.F. SURAI. 2003. Carotenoid modulation of immune function and sexual attractiveness in Zebra Finches. *Science* 300:125–127.
- BOOMSTROM, M.R. AND G. RITCHISON. 2006. Possible relationships between morphology, territory quality, and skin color of American Kestrels. *Journal of Field Ornithology* 4:392–398.
- BORTOLOTTI, G.R., K.J. FERNIE, AND J.E. SMITS. 2003. Carotenoid concentration and coloration of American Kestrels (*Falco sparverius*) disrupted by experimental exposure to PCBs. *Functional Ecology* 17:651–657.
- , J.J. NEGRO, J.L. TELLA, T.A. MARCHANT, AND D.M. BIRD. 1996. Sexual dichromatism in birds independent of diet, parasites and androgens. *Proceedings of the Royal Society of London Series B* 263:1171–1176.
- , J.L. TELLA, M.G. FORERO, R.D. DAWSON, AND J.J. NEGRO. 2000. Genetics, local environment and health as factors influencing plasma carotenoid levels in wild American Kestrels (*Falco sparverius*). *Proceedings of the Royal Society of London Series B* 267:1433–1438.

- BRADBURY, J.W. AND S.L. VEHRENCAMP. 2011. Principles of animal communication. Sinauer, Sunderland, MA.
- BRUSH, A.H. 1981. Carotenoids in wild and captive birds. Pages 539–562 in J.C. Baurenfeind [Ed.], Carotenoids as colorants and vitamin A precursors. Academic Press, New York, NY U.S.A.
- . 1990. Metabolism of carotenoid pigments in birds. *FASEB Journal* 4:2969–2977.
- BUSTAMANTE, J. AND F. HIRALDO. 1989. Post-fledging dependence period and maturation of flight skills in the Black Kite *Milvus migrans*. *Bird Study* 36:199–204.
- CASAGRANDE, S., D. COSTANTINI, A. FANFANI, J. TAGLIAVINI, AND G. DELL'OMO. 2007. Patterns of serum carotenoid accumulation and skin color variation in kestrel nestlings in relation to breeding conditions and different terms of carotenoid supplementation. *Journal of Comparative Physiology B* 177:237–245.
- , D. CSERMELY, E. PINI, V. BERTACCHE, AND J. TAGLIAVINI. 2006. Skin carotenoid concentration correlates with male hunting skill and territory quality in the kestrel (*Falco tinnunculus*). *Journal of Avian Biology* 37:190–196.
- , G. DELL'OMO, D. COSTANTINI, J. TAGLIAVINI, AND T. GROOTHUIS. 2011. Variation of a carotenoid-based trait in relation to oxidative stress and endocrine status during the breeding season in the Eurasian Kestrel: a multi-factorial study. *Comparative Biochemistry and Physiology A* 160:16–26.
- CHEW, B.P. AND J.S. PARK. 2004. Carotenoid action on the immune response. *Journal of Nutrition* 134:257S–261.
- CONSTANTINI, D. AND A.P. MØLLER. 2008. Carotenoids are minor antioxidants for birds. *Functional Ecology* 22:367–370.
- , M. ROWE, M.W. BUTLER, AND K.J. MCGRAW. 2010. From molecules to living systems: historical and contemporary issues in oxidative stress and antioxidant ecology. *Functional Ecology* 24:950–959.
- CRAMP, S. AND K.E.L. SIMMONS. 1980. The birds of the western Palearctic, Vol. 2: Hawks to bustards. Oxford Univ. Press, Oxford, U.K.
- CURIO, E. 1983. Why do young birds reproduce less well? *Ibis* 125:400–404.
- DARWIN, C. 1871. The descent of man, and selection in relation to sex. John Murray, London, U.K.
- DAWSON, R.D. AND G.R. BORTOLOTTI. 2006. Carotenoid-dependent coloration of male American Kestrels predicts ability to reduce parasitic infections. *Naturwissenschaften* 93:597–602.
- DEL HOYO, J., A. ELLIOTT, AND J. SARGATAL. 1994. Handbook of the birds of the world, Vol. 2. New World vultures to guinea-fowl. Lynx Editions, Barcelona, Spain.
- DESROCHERS, A. 1992. Age-related differences in reproduction by European Blackbirds: restraint or constraint? *Ecology* 73:1128–1131.
- ELLEGREN, H. 1996. First gene on the avian W chromosome (CHD) provides a tag for universal sexing of non-ratite birds. *Proceedings of the Royal Society of London Series B* 263:1635–1641.
- FAIVRE, B., A. GREGOIRE, M. PREAULT, F. CEZILLY, AND G. SORCI. 2003. Immune activation rapidly mirrored in a secondary sexual trait. *Science* 300:103.
- FIGUEROLA, J., E. MUÑOZ, R. GUTIÉRREZ, AND D. FERRER. 1999. Blood parasites, leucocytes and plumage brightness in the Cirl Bunting *Emberiza cirlus*. *Functional Ecology* 13:594–601.
- FOLSTAD, I. AND A.J. KARTER. 1992. Parasites, bright males, and the immunocompetence handicap. *American Naturalist* 139:603–622.
- FORERO, M.G., J.A. DONÁZAR, J. BLAS, AND F. HIRALDO. 1999. Causes and consequences of territory change and breeding dispersal distance in the Black Kite. *Ecology* 80:1298–1310.
- GERARD, L., G.L. HAWKINS, AND G.E. HILL. 2012. Delayed plumage maturation and delayed reproductive investment in birds. *Biological Reviews* 87:257–274.
- KNOWLES, S.C., L.S. NAKAWAHE, AND B.C. SHELDON. 2009. Elevated reproductive effort increases blood parasitaemia and decreases immune function in birds: a meta-regression approach. *Functional Ecology* 23:405–515.
- LAAKSONEN, T., J.J. NEGRO, S. LYYTINEN, J. VALKAMA, I. OTS, AND E. KORPIMÄKI. 2008. Effects of experimental brood size manipulation and gender on carotenoid levels of Eurasian Kestrels *Falco tinnunculus*. *PLoS ONE* 3(6): e2374. doi: 10.1371/journal.pone.0002374.
- LANDIS, J.R. AND G.G. KOCH. 1977. The measurement of observer agreement for categorical data. *Biometrics* 33:159–174.
- MARTÍNEZ-PADILLA, J., F. MOUGEOT, L. PÉREZ-RODRÍGUEZ, AND G.R. BORTOLOTTI. 2007. Nematode parasites reduce carotenoid-based signalling in male Red Grouse. *Biology Letters* 3:161–164.
- MATEOS, C. AND J. CARRANZA. 1997. The role of bright plumage in male-male interactions in the Ring-necked Pheasant. *Animal Behaviour* 54:1205–1214.
- MAYNARD SMITH, J. AND D. HARPER. 2003. Animal signals. Oxford Univ. Press, Oxford, U.K.
- MØLLER, A.P., C. BIARD, J.D. BLOUNT, D.C. HOUSTON, P. NINNI, N. SAINO, AND P.F. SURAI. 2000. Carotenoid dependent signals: indicators of foraging efficiency, immunocompetence or detoxification ability? *Avian and Poultry Biology Reviews* 11:137–159.
- MOUGEOT, F. AND B. ARROYO. 2006. Ultraviolet reflectance in the cere of raptors. *Biology Letters* 2:173–176.
- , ———, AND V. BRETAGNOLLE. 2001. Decoy presentations as a means to manipulate the risk of extrapair copulation: an experimental study in a semicolonial raptor, the Montagu's Harrier (*Circus pygargus*). *Behavioral Ecology* 12:1–7.
- MCCULLAGH, P. AND J.A. NELDER. 1989. Generalized linear models. Chapman and Hall, New York, NY U.S.A.
- MCDONALD, P.G. 2003. Variable plumage and bare part colouration in the Brown Falcon, *Falco berigora*: the influence of age and sex. *Emu* 103:21–28.
- MCGRAW, K.J. AND G.E. HILL. 2006. Mechanics of carotenoid-based colouration. Pages 177–242 in G.E. Hill and K.J. McGraw [Eds.], Bird coloration, Vol. 2: Function and evolution. Harvard Univ. Press, Cambridge, MA U.S.A.

- MONTGOMERIE, R. 2006. Analyzing colors. Pages 113 in G.E. Hill and K.J. McGraw [Eds.], *Bird coloration*, Vol. 2: Function and evolution. Harvard Univ. Press, Cambridge, MA U.S.A.
- MUNSELL, A.H. 1912. A pigment color system and notation. *American Journal of Psychology* 23:236–244.
- NEGRO, J.J., G.R. BORTOLOTTI, J.L. TELLA, K.J. FERNIE, AND D.M. BIRD. 1998. Regulation of integumentary colour and plasma carotenoids in American Kestrels consistent with sexual selection theory. *Functional Ecology* 12:307–312.
- , J.M. GRANDE, J.L. TELLA, J.L. GARRIDO, D. HORNERO, J.A. DONÁZAR, J.A. SÁNCHEZ-ZAPATA, J.R. BENÍTEZ, AND M. BARCELL. 2002. Coprophagy: an unusual source of essential carotenoids – a Yellow-faced Vulture includes ungulate faeces in its diet for cosmetic purposes. *Nature* 416:807–808.
- NEWTON, I. AND P. ROTHERY. 2001. Estimation and limitation of numbers of floaters in a Eurasian Sparrowhawk population. *Ibis* 143:442–449.
- PENTERIANI, V., M. FERRER, AND M.M. DELGADO. 2011. Floater strategies and dynamics in birds, and their importance in conservation biology: towards an understanding of nonbreeders in avian populations. *Animal Conservation* 14:233–241.
- PÉREZ-RODRÍGUEZ, L. 2008. Carotenoid-based ornamentation as a dynamic but consistent individual trait. *Behavioral Ecology and Sociobiology* 62:995–1005.
- SARASOLA, J.H., J.J. NEGRO, M.J. BECHARD, AND A. LANUSSE. 2011. Not as similar as thought: sexual dichromatism in Chimango Caracaras is expressed in the exposed skin but not in the plumage. *Journal of Ornithology* 152:473–479.
- SERGIO, F., J. BLAS, R. BAOS, M.G. FORERO, J.A. DONÁZAR, AND F. HIRALDO. 2009b. Short and long-term consequences of individual and territory quality in a long-lived bird. *Oecologia* 160:507–514.
- , ———, G. BLANCO, A. TANFERNA, L. LÓPEZ, J.A. LEMUS, AND F. HIRALDO. 2011a. Raptor nest decorations are a reliable threat against conspecifics. *Science* 331:327–330.
- , ———, M.G. FORERO, J.A. DONÁZAR, AND F. HIRALDO. 2007a. Size-related advantages for reproduction in a slightly dimorphic raptor: opposite trends between the sexes. *Ethology* 113:1141–1150.
- , ———, ———, ———, AND ———. 2007b. Sequential settlement and site dependence in a migratory raptor. *Behavioral Ecology* 18:811–821.
- , ———, AND F. HIRALDO. 2009a. Predictors of floater status in a long-lived bird: a cross-sectional and longitudinal test of hypotheses. *Journal of Animal Ecology* 78:109–118.
- , G. TAVECCHIA, J. BLAS, L. LÓPEZ, A. TANFERNA, AND F. HIRALDO. 2011b. Variation in age-structured vital rates of a long-lived raptor: Implications for population growth. *Basic and Applied Ecology* 12:107–115.
- STEENHOF, K. AND B. PETERSON. 2009. Site fidelity, mate fidelity, and breeding dispersal in American Kestrels. *Wilson Journal of Ornithology* 121:12–21.
- STERNALSKI, A., F. MOUGEOT, AND V. BRETAGNOLLE. 2012. Carotenoid limitation and allocation priorities in asynchronous raptor nestlings. *Biological Journal of the Linnean Society* 105:13–24.
- , ———, C. ERAUD, B. GANGLOFF, A. VILLERS, AND V. BRETAGNOLLE. 2010. Carotenoids in nestling Montagu's Harriers: variations according to age, sex, body-condition and evidence for diet-related limitations. *Journal of Comparative Physiology B* 180:33–43.
- SUDD, M.V. AND R.J. ROBERTSON. 1985. Evidence for reliable badges of status in territorial Yellow Warblers (*Dendroica petechia*). *Animal Behaviour* 33:1102–1113.
- VERGARA, P. AND J.A. FARGALLO. 2011. Multiple coloured ornaments in male common kestrels: different mechanisms to convey quality. *Naturwissenschaften* 98:289–298.
- VILLAGE, A. 1985. Turnover, age and sex ratios of kestrels (*Falco tinnunculus*) in south Scotland. *Journal of Zoology* 206:175–189.
- WIERSMA, P., C. SELMAN, J.R. SPEAKMAN, AND S. VERHULST. 2004. Birds sacrifice oxidative protection for reproduction. *Biology Letters* 271:S360–S363.
- ZACK, S. AND B.J. STUTCHBURY. 1992. Delayed breeding in avian social systems: the role of territory quality and “floater” tactics. *Behaviour* 123:194–219.
- ZAHAVI, A. 1975. Mate selection—a selection for a handicap. *Journal of Theoretical Biology* 53:205–214.
- ZUUR, A.F., E.N. LENO, N.J. WALKER, A.A. SAVELIEV, AND G.M. SMITH. 2007. Mixed effects models and extensions in ecology with R. Springer, New York, NY U.S.A.

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