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ORIGINAL PAPER

Beak colour reflects circulating carotenoid and vitamin A levels in spotless starlings (*Sturnus unicolor*)

Carlos Navarro · Tomás Pérez-Contreras · Jesús M. Avilés · Kevin J. Mcgraw · Juan J. Soler

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Abstract Many colourful sexually selected signals in animals are carotenoid-dependent and, because carotenoids function as antiradicals and immunostimulating molecules, carotenoid-dependent signals may honestly reflect the health state of individuals. Some others nutrients like vitamin A may also enhance health and colouration, but these have rarely been tested alongside carotenoids in colourful birds. Here, we examined whether beak colour of the spotless starling (*Sturnus unicolor*) reflected circulating levels of carotenoids and/or vitamin A (retinol). Spotless starlings are polygynous, sexually dimorphic birds (i.e. length of chest feathers). The tip of the beaks of male and female spotless starlings is more intensely coloured at the beginning of the breeding season and becomes dull after mating, which may suggest a sexual function. We found that females have a more intensely coloured beak and higher plasma carotenoid concentration than males during mating, and, despite the finding that carotenoid and vitamin A levels were not significantly related; colour intensity was positively correlated with plasma concentration of carotenoids and vitamin A in both sexes. However, adult beak coloration was not associated with carotenoid and vitamin A concentrations after nestlings were hatched. Therefore, beak colouration of spotless starlings provides information about circulating levels of carotenoids and vitamins during the mating season and may potentially function as a reliable signal of physiological status in the context of sexual selection.

Keywords Antioxidant · Beak colouration · Plasma carotenoids · Retinol · Sex differences · Plastic signals · Vitamin A

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C. Navarro (✉) · T. Pérez-Contreras · J. M. Avilés · J. J. Soler
Departamento de Ecología Funcional y Evolutiva,
Estación Experimental de Zonas Áridas (CSIC),
E-04120 Almería, Spain
e-mail: cnavarro@eeza.csic.es

J. M. Avilés
Departamento de Biología Animal y Ecología,
Universidad de Granada,
E-18071 Granada, Spain

K. J. Mcgraw
School of Life Sciences, Arizona State University,
Tempe, AZ 85287-4501, USA

Introduction

Signals are any acts or structures that alter the behaviour of other organisms and often benefit both communicating parties (Endler 2000; Maynard Smith and Harper 2003). Zahavi (1975) proposed that signals should reliably indicate phenotypic quality of individuals by means of differential costs associated with signal production or maintenance. Only high-quality individuals could afford such costs, which therefore assure the honesty of the signal. Honest signals may act in many different contexts, such as social status signalling (e.g. Møller 1987; McGraw and Hill 2000) or sexual selection (e.g. Møller 1988; Petrie 1994).

Carotenoids are one of the most important pigments responsible for colouration in birds (e.g. skin, feathers), and in the last few decades carotenoids have attracted the attention of many behavioural ecologists as potential honest signals of individual quality (Olson and Owens 1998; McGraw 2006). Much of the interest in carotenoids centers on the fact that they cannot be synthesised *de novo* by

animals; rather, they must be acquired from plants and herbivores in the diet (Kodric-Brown 1989; Hill 1991; Olson and Owens 1998). Consequently, carotenoid-based colouration should be linked to an individual's capacity to acquire food rich in carotenoids and to assimilate and process those nutrients (Endler 1980; Hill 1999).

Carotenoids may also improve individual health through their free-radical-scavenging actions (Burton and Ingold 1984; Møller et al. 2000; Chew and Park 2004; Martinez et al. 2008). However, Costantini and Møller (2008) recently showed in a meta-analysis of all available studies that there was no evidence that carotenoids are important antioxidants in birds. Carotenoids have also been touted as valuable immunostimulants, perhaps via gene-regulatory or cell-communication mechanisms (Møller et al. 2000), further promoting their honesty in colour signalling. Experimental studies manipulating dietary carotenoid levels (Blount et al. 2003; McGraw and Ardia 2003) and immunocompetence (Faivre et al. 2003; Alonso-Alvarez et al. 2004) have produced results consistent with this view.

In addition to the focus on health actions of carotenoids, Hartley and Kennedy (2004) recently proposed that some vitamins (e.g. C, E, A) and enzymes with better antioxidant power than carotenoids would ultimately protect carotenoids from oxidation, permitting their use as honest colour signals. Several experimental studies have shown a positive relationship between non-pigmentary antioxidants (e.g. melatonin, vitamin E) and colouration in birds and fishes (e.g. Bertrand et al. 2006; Pérez et al. 2007; but see Karu et al. 2008), but no work to date has examined natural covariation among such non-pigmentary antioxidants, circulating carotenoids and colouration in free-living animals.

Here, we focused on vitamin A, which is a fat-soluble vitamin that plays key roles in vision, reproduction, growth and development and can be ingested directly from animal tissue or formed from carotenoid (e.g. β-carotene) precursors. Absorption efficiency of vitamin A depends on fat content of the diet (see review in Debier and Larondelle 2005), and its antioxidant and immune-boosting properties are also well recognised (Friedman and Sklan 1997). Aside from applied research in domestic poultry (e.g. Kuenzel et al. 2006) and some work on the retinol composition of tissues in free-living seabirds (Wallace et al. 1996; Surai et al. 2000), very little is known about the antioxidant and morphological effects of this vitamin in wild birds.

Females may rely on carotenoid-based ornaments as direct indicators of male condition and male capacity to acquire nutrients (Andersson 1994; Hill 2002). Furthermore, females may also use carotenoid-dependent characters to signal their own condition, and, if both males and females use the same kind of signals, mutual mate preferences are expected in species with biparental care (Burley 1986) such as the spotless starling (*Sturnus unicolor*; Soler et al. 2008).

The majority of historical work on carotenoid-based honest signals in birds has focused on plumage (Hill 2006); only in a few taxa have the carotenoid-based colourful bare parts in adult birds been closely examined (e.g. zebra finches, blackbird, partridge, grouse, boobies; Burley and Coopersmith 1987; Faivre et al. 2003; Perez-Rodriguez and Viñuela 2008; Martinez-Padilla et al. 2007; Velando et al. 2006). The beak of birds can be pigmented by melanin (e.g. horn, black, grey) and carotenoid (e.g. red, orange, yellow) pigments. For instance, astaxanthin, α-doradexantin, adonirubin and canthaxanthin are characteristic pigments of red bird beaks, but are not present in the plasma of the birds and, consequently, are likely directly metabolised in the beak (see McGraw 2004). Unlike some carotenoid-dependent feather ornaments, which are dead tissues, those that are present in bare-part structures like skin or beak must be mobilised continuously (Lozano 1994). Therefore, carotenoid-based signals in bare parts can better indicate real-time physiological status, body condition and resource availability, and consequently such signals may be used by individuals of both sexes to assess the current breeding state of a mate (e.g. Morales et al. 2009; Velando et al. 2006). This scenario predicts that within-individual variation in physiological status (i.e. carotenoid/antioxidant accumulation internally) is associated with intra-individual variation in carotenoid-based colouration across the breeding season.

Here, we examine relationships between carotenoid-dependent beak colour and circulating carotenoid and vitamin A levels across a breeding season in an avian species with biparental care—the spotless starling. The spotless starling is a polygynous species (Veiga et al. 2001) that in our population typically lays clutches of four to five eggs that, with a few exceptions, are incubated by females (Soler et al. 2008). Male throat-feather length is positively correlated with mating success in this species, suggesting a sexually selected role (Aparicio et al. 2001). Additionally, the end of the beak in males is yellow, turning grey with a tone of blue–green at the base. The end of the beak in the female is yellow, but pink at the base (Fig. 1). After breeding, the beak loses brilliant colouration and becomes dusky or even black in winter (Cramp 1998), which may suggest a sexual role before the reproduction.

The aims of this study were to (1) test for sexual differences in yellow beak colouration of spotless starlings; (2) investigate the relationships between beak colouration and plasma carotenoid levels, plasma vitamin A levels and body condition of males and females and (3) examine changes in the association between beak colouration and plasma carotenoids, vitamin A concentrations, and body condition across the breeding season. Our initial predictions are that; (1) males and females should differ in beak

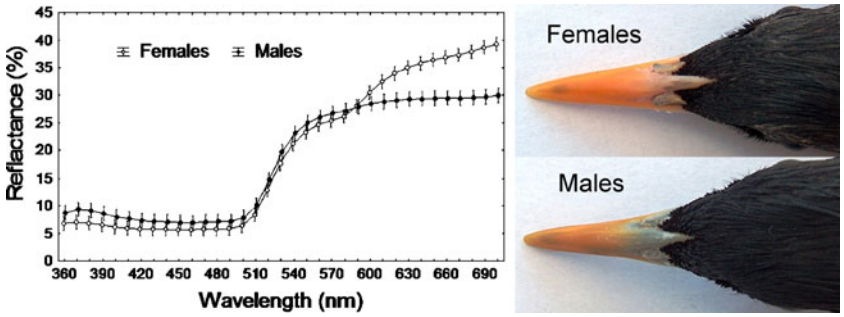

Fig. 1 Mean spectral reflectance of male (*filled triangles*) and female (*empty triangles*) spotless starling beaks. *Vertical bars* denote 95% confidence intervals. Photographs of the beak of male and female starlings are also shown

colouration intensity, (2) beak colouration is positively related with physiological condition in males and females and (3) because males and females are exposed to different physiological demands at different reproductive stages (e.g. nest defence, egg laying, incubation, nestling feeding), the predicted association between beak colouration and physiological status should differ for males and females.

Methods

Study area and capture of birds

This study was carried out in Guadix (southeastern Spain—37°18′N, 3 ° 11′W) in March–June 2007. We captured and ringed 64 spotless starlings (30 males and 34 females) at daybreak (6:30–8:30 a.m.) inside nest boxes (23×23×35 cm) installed close to or within colonies already established in old buildings in the area.

Bird were capture at the beginning of the breeding season (20 March–14 April 2007), when starlings are pairing and building nests. At the beginning of the nestling stage (2–3 days post-hatch), we captured 41 reproductive females and 20 reproductive males. Also, 13 males and nine females were captured at the beginning of the breeding season and after hatching of their eggs. We determined body mass (pesola, accuracy of 0.5 g) and tarsus length (digital calliper, accuracy of 0.01 cm) for all birds. Body condition was calculated as residuals from the mass-tarsus regression. Finally, by puncturing the brachial vein, we collected 50 μl of blood in heparinized tubes within the first two minutes after capture, thereby reducing the effect of stress on plasma antioxidants (Romero and Reed 2005; Chastel et al. 2005). Blood samples were placed on ice (4–5°C) until centrifugation and plasma was frozen immediately afterwards (at −20°C).

Vitamin A and carotenoid analyses

We analysed retinol (vitamin A; absorbance maximum = 325 nm) and carotenoid levels following the protocol described in McGraw et al. (2008). Briefly, we thawed and added 15 μl of plasma to a microcentrifuge tube that together with 100 μl of ethanol was vortexed for 5 s. Afterward, we added 100 μl of MTBE and vortexed again for 5 s. We then centrifuged tubes for 3 min at 12,000 rpm. We transferred the supernatant to a fresh screw cap Eppendorf tube and evaporated to dryness with a nitrogen evaporator in a hood. Next, we resuspended the supernatant in 200 μl mobile phase, vortexed for 5 s, and injected 50 μl into a high-performance liquid chromatograph (HPLC; Waters Alliance® Instrument, Waters Corporation, Milford, MA). We used a 5 μm Waters Carotenoid C-30 column (4.6×250 mm ID) to determine types and amounts of carotenoids present. Pigment concentrations were calculated based on external curves constructed from known amounts of purified reference carotenoids. All measurements were made by CN, which reduced variance due to inter-observer variability, and after obtaining repeatable measurements in preliminary tests. Analyses were performed blind with respect to sex and beak. Based on comparison to reference carotenoids, we identified and estimated concentrations of cis-lutein, trans-lutein, zeaxanthin, β-carotene and esterified carotenoids (Table 1). We summed all concentrations to determine total carotenoid levels in plasma.

Beak colour measurements

Though avian visual perception can be now modelled in complex ways, incorporatingretinal sensitivities and ambient wavelengths (Endler et al. 2005; Avilés et al. 2008), here we relied on colour variables extracted from spectrophotometric data that have proved to successfully reveal individual quality in previous work (see review in Hill and McGraw 2006). Beak colour was measured three times (except one bird that was measured only twice) on the distal yellow part (see Fig. 1). Reflectance spectra were obtained from 360–700 nm for all birds just after blood sampling using a spectrophotometer (Konica Minolta Sensing [Seoul, South Korea], CM-2600d) that estimates values at 10-nm intervals. Beaks were illuminated at 90º to the measuring

Table 1 Means and standard deviation for plasma concentrations of vitamin A and carotenoids (cis-lutein, trans-lutein, zeaxantin, β-carotene and esterified carotenoids) in wild male and female spotless starlings

Antioxidant	Male=30 Mean (SD)	Female=34 Mean (SD)	$F_{1,60}$	p
Cis-lutein	0.729 (0.099)	0.654 (0.051)	0.18	0.67
Trans-lutein	4.875 (0.478)	6.281 (0.412)	4.39	0.04
Zeaxantin	0.684 (0.088)	0.728 (0.040)	0.05	0.82
β-carotene	0.032 (0.004)	0.041 (0.003)	1.89	0.17
Carotenoid esters	0.161 (0.017)	0.226 (0.015)	6.81	0.01
Vitamin A	0.920 (0.197)	0.903 (0.186)	0.15	0.69

Results of univariate comparisons (F_{df} and associated p values) between sexes for all carotenoids and vitamin A are also shown

surface by a xenon light source, and the reflected light captured at the same angle. The measurements were taken relative to standard white (CM-A145, Konica Minolta Sensing) and dark references (CM-A32, Konica Minolta Sensing), which we calibrated before measurement of each bird. From spectrophotometric data, we calculated brightness as mean reflectance at every 10 nm interval from 360–700 nm. The shape of the spectrum of the beak of females shows two peaks. The first one corresponds with part of the range of absorbance of carotenoids (i.e. 450–570 nm; Britton et al. 1995), whereas the second (570–700 nm) coincides with the typical gradual increasing spectral shape of phaeomelanins (Prota 1992; Riley 1997; Fig. 1). This second peak, however, does not appear in the spectrum of males (Fig. 1).

Because we aimed to study sexual differences in the carotenoid waveband, we estimated hue and chroma within the 450–570 nm waveband for males and females. Hue was calculated as the wavelength at the inflection point of the spectra within the different wavelength ranks (Keyser and Hill 1999). Hue is a good indicator of the carotenoid pigment concentration of the integument in some species (Saks et al. 2003). Carotenoid chroma was defined as the proportion of reflectance within the carotenoids waveband (450-570 nm; Peters et al. 2004) in relation to total reflectance (360–700 nm). Birds that had high chroma values for the 450–570 nm interval had low chroma values for the 570–700 nm interval (see Fig. 1), so chroma estimates for low wavelength (450–570) were not used in our analyses.

Multiple measures of coloration in each sampled bird (see above) were repeatable both in females (brightness: r=0.75, $F_{82.165}$=7.03, p<0.0001; hue $_{(450–570nm)}$: r=0.63, $F_{82.165}$=4.45, p<0.0001; chroma $_{(450,570nm)}$: r=0.94, $F_{82.165}$=35.77, p<0.0001) and males (brightness: r=0.70, $F_{58.118}$=5.85, p<0.0001; hue $_{(450–570nm)}$: r=0.47, $F_{58.}$=2.77, p<0.0001; chroma $_{(450–570nm)}$: r=0.94, $F_{58.118}$=29.56, p<0.0001) Therefore, we used average values per individual for statistical analyses (Sokal and Rohlf 1981; Zar 1984).

All data, except hue values, approximately followed a normal distribution (Kolmogorov–Smirnov test for continuous variables, p>0.2). One outlier colour value for a female (Brightness=469.82; >2 standard deviation [mean=670.02; SD=95.78]) was eliminated from our data set. We examined intersexual differences in beak colour measures, body condition and plasma levels of retinol and carotenoids using measures obtained at the first capture (30 males and 33 females). We used capture date and the squared capture date as covariates in statistical analyses since plasma levels of vitamin A and carotenoids changed during the season following a quadratic function (multivariate analyses of variance (MANOVA), Date $F_{2,95}$=3.32, p=0.04; Date^2, $F_{2,95}$=4,96, p=0.008) (see Ninni et al. 2004).

MANOVA tests were used to assess relationships between beak colouration (dependent variables) and variables related to plasma antioxidants (total plasma carotenoid level (TPCL) and vitamin A level) and body condition as continuous independent variables. Sex was included as a fixed independent factor in the analysis. After estimation of statistical parameters associated with the above factors, we included in the model the interaction terms between sex and the continuous independent variables to explore sexual differences in the relationships between beak colour and plasma level of carotenoids and vitamin A (see Quinn and Keough 2002). The association between colour variables for the beak and plasma antioxidants and body condition was also explored for birds captured 2–3 days after hatching. In this analysis, hatching date was included as an additional covariate to correct for possible seasonal effects. Finally, for individuals captured twice (i.e. during mating and subsequently after hatching), we analysed within-season differences in the association between beak colouration and plasma antioxidants. In this analysis, we subtracted the average hatching date in our population from the capture date that was entered in the model to correct for possible seasonal trends. Two tailed p values and alpha level of 0.05 were used for statistical inference.

Results

Sexual differences

We found sex differences in beak-tip reflectance within the carotenoid waveband (i.e. 450–570 nm; MANOVA: $F_{3.57}$=48.14 p<0.0001), after controlling for the effect of date (MANOVA: $F_{3.57}$=11.69 p<0.0001) and squared date

(MANOVA: $F_{3.53}$=12.05 p<0.0001). Female beaks were more saturated ($F_{1.59}$=67.57, p<0.0001) and showed a non-significant trend to be brighter ($F_{1.59}$=3.32, p=0.051) than those of males in the carotenoid waveband (i.e. 450–570 nm; Fig. 2). The hue within the carotenoid waveband did not vary with sex ($F_{1.59}$=0.26, p=0.61). Moreover, males had lower circulating carotenoids in plasma than females (MANOVA test for differences in total carotenoid concentration between males and females, including capture date and capture date squared as covariates; Sex effect: $F_{5.56}$=2.58, p=0.036, see univariate test results in Table 1). Finally, we did not detect a sex difference in plasma vitamin A concentration ($F_{1.60}$=0.15, p=0.69).

Correlations among body condition, circulating antioxidants and beak colour during the mating period

During the mating season, we found a significant positive relationship between carotenoid hue of the beak tip and TPCL in both sexes (Table 3). We also detected negative associations between carotenoid chroma and brightness of the beaks and TPCL after controlling for the effect of sex and date, and date squared (MANOVA: $F_{3.53}$=19.56 p<0.0001, see univariate test result in Table 2, Fig. 3). Plasma level of vitamin A also explained additional variation in beak colour (i.e. chroma; MANOVA: $F_{3.53}$=2.90, p=0.04; see results of univariate tests in Table 2, Fig. 3). Furthermore, the relationships between beak colour and TPCL, but not those between beak colour and body condition and vitamin A, differed for males and females during the mating season (MANOVA: Sex×TPCL: $F_{3.52}$=3.71, p<0.017; Sex xvitamin A: $F_{3.52}$=0.92, p<0.43; Sex×body condition: $F_{3.52}$ = 2.23, p<0.09, see results of univariate tests in Table 2). The relationship between beak brightness and TPCL was positive in females, but negative in males (Table 3). We also found that the slope of the relationship between hue and TPCL was steeper in males than in females (Fig. 3b; see statistics associated to the interaction term in Table 3). Both males and females show a negative relation between TPCL and carotenoid chroma estimated for low wavelengths. Plasma levels of vitamin A were not related to TPCL ($F_{1.61}$=2.00, p=0.16). In addition, there was no significant relationship between body condition and TPCL ($F_{1.59}$= 0.38, p=0.13) or vitamin A ($F_{1.59}$=0.40, p=0.52) after controlling for sex, capture date and squared capture date square.

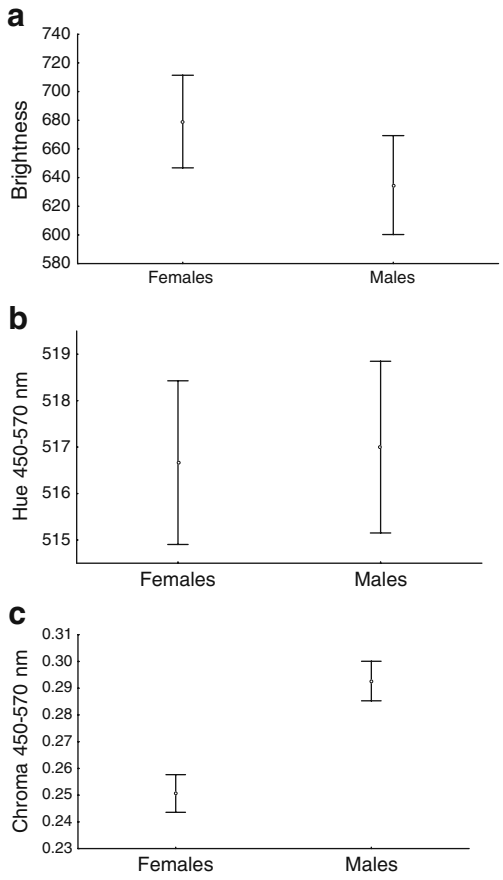

Fig. 2 Means±SE values of the tip of beak brightness (**a**), hue (450–570 nm) (**b**) and chroma (450–570 nm) (**c**) for male (n=30) and female (n=33) spotless starlings

Correlations among body condition, circulating antioxidants and beak colour after hatching

Neither body condition, TPCL, nor vitamin A explained colour variables of the beak tip when we analysed these relationships with individuals caught a few days after hatching (i.e. chroma (450–570), hue (450–570) and brightness; MANOVA: Body condition $F_{3.46}$=0.35, p=0.79; TPCL $F_{3.46}$=0.25, p=0.86; Vitamin A $F_{3.46}$=0.71, p=0.55), after controlling for the effect of sex (MANOVA: $F_{3.46}$=8.88, p<0.001), hatching date (MANOVA: $F_{3.46}$=0.67, p=0.57) and squared hatching date (MANOVA: $F_{3.46}$=0.90, p=0.45). The interaction terms between sex and TPCL (MANOVA: $F_{3.45}$=2.05, p=0.12), sex and vitamin A (MANOVA: $F_{3.45}$= 1.25, p=0.30) and sex and body condition (MANOVA: $F_{3.45}$=0.45, p=0.71) did not explain a significant proportion of variance in beak colouration 2–3 days after hatching. Only the interaction between sex and squared date was significant (MANOVA: $F_{3.45}$=2.72, p=0.05). Univariate tests showed that the relationship between hatching date and chroma (450–570) ($F_{1.47}$=8.05, p=0.007), but not for hue (450–570; $F_{1.47}$=3.33, p=0.07) or brightness ($F_{1.47}$= 0.06, p=0.80), varied with sex. Chroma (450–570) increased as the season progressed in females ($F_{1.35}$=35.61, p<0.0001, r=0.71) but not in males ($F_{1.16}$=0.09, p=0.76, r=−0.08).

Table 2 MANOVA test with bill brightness, hue (450–570 nm) and chroma (450–570 nm) as dependent variables. Sex was used as a fixed factor and capture date, capture date squared (date^2), body condition, vitamin A and total carotenoid plasma level (TCPL) as continuous variables

				Chroma (450–570nm)		Hue (450–570nm)		Brightness	
Variables	Wilks's values	$F_{3,53}$ $F^*_{3,52}$	p	Beta (SE)	p	Beta (SE)	p	Beta (SE)	p
Sex	0.32	37.21	<0.001	−0.56 (0.07)	<0.001	−0.22 (0.13)	<0.093	0.41 (0.14)	0.005
Date	0.74	6.06	0.001	−1.07 (0.46)	0.025	1.60 (0.84)	0.063	2.55 (0.93)	0.008
Date2	0.72	6.78	<0.001	1.05 (0.45)	0.024	−1.57 (0.82)	0.060	−2.75 (0.90)	0.003
Body condition	0.96	0.63	0.596	0.03 (0.07)	0.669	−0.07 (0.13)	0.545	0.19 (0.14)	0.183
Vitamin A	0.85	2.90	0.043	0.16 (0.06)	0.010	−0.03 (0.11)	0.777	0.20 (0.12)	0.108
TPCL	0.47	19.56	<0.001	−0.49 (0.06)	<0.001	0.556 (0.12)	<0.001	−0.36 (0.13)	0.008
Sex×body condition	0.89	2.23	0.095	0.09 (0.10)	0.379	0.58 (0.12)	0.176	0.45 (0.19)	0.020
Sex * Vitamin A	0.95	0.93	0.435	−0.17 (0.30)	0.561	0.36 (0.54)	0.504	−0.96 (0.58)	0.106
Sex * TPCL	0.82	3.72	0.017	0.54 (0.17)	0.003	−0.56 (0.33)	0.09	0.64 (0.36)	0.082

The effects of interaction terms between sex and continuous variables were subsequently estimated in separated models that also included the independent variables and therefore degrees of freedoms associated to F values change ($F_{3,53}$ for estimates of independent variable effects and $F^*_{3,52}$ for estimates of the effects of the interaction terms). Univariate results (Beta (standard error) and associated p values) for brightness, hue (450–570 nm) and chroma (450–570 nm) are also shown. All results are consistent and retain significance after removing non-significant interactions from the initial model

Temporal changes in beak colour, body condition and antioxidant levels

The analysis of beak colouration on recaptured birds revealed that beak colouration was plastic in both male (MANOVA $F_{4,45}$=8.9, p=0.00002) and female (MANOVA $F_{4,69}$=8.7, p=0.00001) spotless starlings. Beaks of females at the hatching period were less bright, had lower hues and were more saturated at the carotenoid waveband beaks compared to those in the mating period. Beaks of males captured two days after hatching had lower brightness and chroma (450–570 nm) but higher green–yellow hue (450–570) than those during the mating period (see Table 4).

Furthermore, for males, TPCL ($F_{1,48}$=8.74, p=0.005) and vitamin A ($F_{1,48}$=7.36, p=0.009) were higher during the nestling (TPCL, Mean (SE)=9.20 (0.64)); Vitamin A: Mean (SE) = 1.09 (0.05)) than during the mating stage (TPCL, Mean (SE) = 6.43 (0.58); Vitamin A: Mean (SE) = 0.93 (0.04)). Similarly, females showed an increase in vitamin A concentration ($F_{1,68}$=6.99, p=0.01; mating stage: Mean (SE) = 0.90 (0.03), post-hatching stage: Mean (SE) = 1.03 (0.04)), but a reduction of plasma carotenoid level ($F_{1,68}$=11.96, p=0.0009; mating stage: Mean (SE) = 7.83 (0.48), nestling stage: Mean (SE)=5.22 (0.47)) from mating to hatching.

Discussion

The main findings of this study were that (1) female spotless starlings had more colourful beaks and higher plasma concentration of carotenoids than males, but not a higher concentration of vitamin A; (2) beak colouration was associated with total plasma carotenoid and plasma vitamin A levels at the onset of mating, but not during the nestling phase, both in males and females; (3) beak colouration was a plastic trait that changed differently in males and females across the different reproductive stages (i.e. from mating to post-hatching) and (4) from mating to post-hatching stages, plasma concentration of vitamin A increased in both sexes, but carotenoids increased in males and decreased in females. Below, we discuss these results that are consistent with the hypothesis that beak colouration is a dynamic signal that reflects changing physiological status (Safran et al. 2008; Rubenstein and Hauber 2008).

Male birds typically have more colourful carotenoid-dependent characters than females (Price and Burley 1993; McGraw et al 2003; Faivre et al. 2003; McGraw and Ardia 2005). However, we found that female starlings had higher levels of circulating carotenoids than males. This sex difference cannot be attributed to blood plasma-serum ratio since males and females did not differ in hematocrit estimations (unpublished data of the same starling population from 2005 [male (n=61), mean=52.9%, SD=3.2; female (n=41), mean=52.2%, SD=2.7] and 2006 [male (n=35), mean=52.4%, SD=3.1.; female (n=48), mean=52.1%, SD=2.8], $F_{1,183}$=1.77, p=0.18), even after controlling for date ($F_{1,182}$=0.017, p=0.90) and date square ($F_{1,81}$=0.074, p=0.79). Beak tips of male and female spotless starlings did not differ in hue estimates for carotenoid wavelengths. However beak tips of females show an additional crest at longer wavelengths (570–

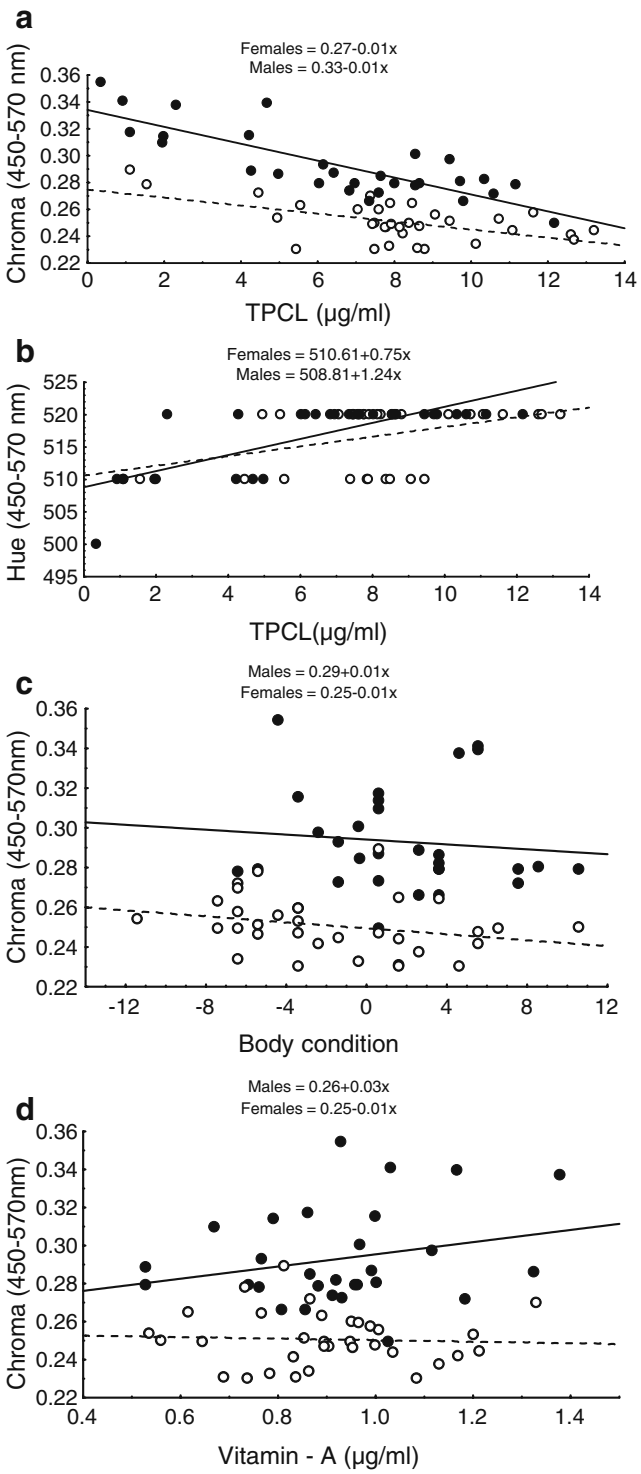

Fig. 3 Relationships between plasma carotenoid concentration and **a** beak chroma and **b** hue; **c** between body condition and beak chroma; and **d** between plasma vitamin A concentration and beak chroma. Data points for males (filled symbols) and females (open symbols) and regression lines are shown

700 nm) (Fig. 1) and, thus, the beak tips of females are more colourful than those of males (Fig. 1). These detected sexual differences may suggest that this character is subject to more intense selection in females than in males. The spotless starling, however, is a polygynous species and, although social polyandry has been detected in our population (Navarro et al., unpublished data), higher intensity of sexual selection in females is an unlikely explanation for the detected sexual dimorphism in beak colouration. Female acquisition and maintenance of breeding sites is closely related to their breeding success, and because steroid-hormone implants affect these abilities (Veiga and Polo 2008) it is likely that other traits showing phenotypic quality also covaried with the ability of females to acquire and maintain breeding sites. Beak colour intensity would thus be a good candidate for signalling in a female-female competition context.

Independent of whether sexual selection is stronger for beak coloration in males or females, males selecting females with colourful beaks are the most likely cause of the evolution of exaggerated carotenoid-based colouration of female beaks (Amundsen and Pärn 2006, but see Rubenstein and Lovette 2009 for cooperative breeding bird species). It is known that females transfer essential micronutrients, including carotenoids, vitamin A and immune factors, to the offspring and that the amount of such substances should be related to females' phenotype and physiological abilities (i.e. availability in the female body; e.g. Morales et al. 2006; Biard et al. 2007). If that was the case, spotless starling males may select females with more colourful beaks because they have higher plasma concentration levels of carotenoids and vitamin A, and it is expected that those females transfer to the eggs larger amount of those substances than others. Reciprocally, by evaluating beak colour intensity of males, females may also estimate male health and condition, since the relationship between colouration and plasma concentrations of carotenoids and vitamin A exist in both sexes.

A relationship between beak colour measures and total plasma carotenoid concentration during the mating season has been detected in other avian species (e.g. Faivre et al. 2003; McGraw et al. 2003). Mechanistically, this relationship could be explained by concentration-dependent uptake of carotenoids from blood circulation by rhamphothecal keratinocytes, (Lucas and Stettenheim 1972). Furthermore, because the possible influence of sex steroid hormones on carotenoids and colouration (Alonso-Alvarez et al. 2004), such a relationship may differ for males and females; accordingly, we found that the relationship between beak colour and plasma carotenoid concentration differed significantly between males and females, with a tighter relationship for males (Table 3). In addition to the fact that physiological state influences sexual signal expression, we

Table 3 Slopes and associated p values of regression analyses between beak colour variables (chroma, hue and brightness) and date, date square, body condition, vitamin A plasma level and TPCL for males and females

	Tip chroma (450–570nm)				Tip Hue (450–570nm)				Tip brightness			
	Female (n=33)		Male (n=30)		Female (n=33)		Male (n=30)		Female (n=33)		Male (n=30)	
	Slope (SE)	p	Slope (SE)	p	Slope (SE)	p	Slope (SE)	p	Slope (SE)	p	Slope (SE)	p
Date	0.0004 (5.97)	0.38	−0.002 (3.74)	0.03	−0.11 (5.98)	0.42	0.38 (3.90)	0.13	−4.06 (5.81)	0.12	−0.04 (4.08)	0.12
Date ^{2}	0.91×10^{-5} (366.44)	0.18	-4.58×10^{-5} (196.90)	0.04	−0.003 (369.72)	0.27	0.01 (204.98)	0.15	−0.08 (354.29)	0.05	0.01 (213.63)	0.93
Body condition	−0.0008 (4.91)	0.14	−0.0006 (4.22)	0.60	0.02 (5.08)	0.90	0.24 (4.17)	0.34	5.20 (4.85)	0.09	−1.75 (4.23)	0.71
Vitamin A	−0.004 (0.19)	0.77	0.03 (0.19)	0.17	5.99 (0.19)	0.18	−3.78 (0.20)	0.46	−11.09 (0.19)	0.89	172.63 (0.19)	0.06
TCPL	−0.003 (2.28)	<0.001	−0.006 (1.95)	<0.001	0.75 (2.49)	0.01	1.23 (2.20)	>0.001	7.69 (2.67)	0.18	−14.05 (2.98)	0.01

Table 4 Means and standard deviation for beak colouration (chroma, hue and brightness) in male (MANOVA, $F_{4,45}$=8.9, p=0.00002) and female (MANOVA $F_{4,69}$=8.7, p=0.00001) spotless starlings

Sex	Colour variable	Mating Mean	n=33 n*=30 SD	Feeding Mean	n=41 n*=20 SD	$F_{1.72}$ $F^*_{1.48}$	p
Females	Brightness	680.01	89.52	601.97	140.26	7.70	0.007
	Chroma 450−570 nm	0.25	0.02	0.27	0.02	9.54	0.002
	Hue 450−570 nm	516.67	4.78	512.68	5.49	10.8	0.001
Males	Brightness	640.01	99.10	520.65	122.39	17.45	<0.001
	Chroma 450−570 nm	0.29	0.02	0.27	0.01	14.51	<0.001
	Hue 450−570 nm	517.00	5.35	520.50	2.23	7.6	0.008

Results of univariate comparisons ($F_{1.72}$ for females and $F^*_{1.48}$ for males and associated p values) for beak colour variables estimated at different reproductive stages (i.e. mating and nestling) are also shown

cannot rule out the possibility in our correlational study that signal expression itself (and behavioural responses by signal receivers) feeds back to affect physiological parameters (Safran et al. 2008).

We found for the first time a statistical link between retinol and integumentary colouration. Research effort to date has focused on associations between vitamins E and C and secondary sexual traits. However, vitamin A is metabolically linked to carotenoids (Debier and Larondelle 2005) and can affect sexual activity (Gaál and Csaba 1998) as well as integument development (see review in Debier and Larondelle 2005). Therefore, vitamin A might be involved in the expression of secondary sexual characters. An experimental study is now needed to understand whether or not vitamin A causally impacts carotenoid-based beak colouration in birds.

Concentrations of plasma vitamin A and carotenoids were not inter-correlated, which suggests different nutritional or metabolic mechanisms underlying the accumulation of the two biochemicals. Plasma carotenoid concentration is mainly related to the amount of carotenoids consumed in the diet, and changes in dietary intake can very quickly result in changes in plasma carotenoids (review in Thurnham and Northrop-Clewes 1999, but see Hõrak et al. 2004). Consuming a rich carotenoid diet may result in an increase in plasma retinol, since approximately 10% of consumed carotenoids are in fact pro-vitamin A (i.e. β-carotene) that is transformed to retinol in the intestinal mucosa (Ganguly et al. 1953; Surai 2002). Plasma concentrations of retinol, however, are homeostatically regulated and should remain relatively constant. High or low plasma levels of vitamin A might have serious negative effects, but excess of vitamin A in stored in the liver and released directly to the blood when plasma concentration of vitamin A decreases (Thurnham and Northrop-Clewes 1999; Debier and Larondelle 2005). Consequently, variation in plasma levels of carotenoids and vitamin A could be related to different physiological activities and therefore reveal different aspects of physiological condition of individuals. Independent of the proximate (i.e. physiological) mechanisms explaining the associations between plasma antioxidants and beak colour in spotless starlings, our results suggest that beak colour of both males and females may be used by conspecifics as a signal of physiological status.

Beak colour of male and female spotless starlings changed during the breeding season, suggesting that males or females could mutually and continuously evaluate current mate quality (see Velando et al. 2006). Unlike some carotenoid-dependent ornaments with no physiological activity like feathers (McGraw 2006), carotenoids that are present in beak or other fleshy (i.e. physiological active) integuments can be remobilized continuously and may better indicate ongoing physiological activities. Beak colour changes were different in males and females, and this may reflect sexual differences in hormonal activity (Burley et al. 1992; Negro et al. 1998), costs of reproduction (egg laying, incubation, feeding effort, etc.) across the season and/or ectoparasitism (Ewen et al. 2009). In any case, the expected decrease in carotenoids concentration only occurred in females, while vitamin A increased from mating to hatching in the two sexes. So, sexual mismatches between changes in beak colour and carotenoid levels may suggest that maintenance of such physiologically important chemicals in the integument (i.e. beak) after pairing is no longer adaptive for feeding males, but for females trying to affect feeding effort of their mates (i.e. beak colour as a postmaiting sexually selected signals that affect investment in reproduction of her male). This possibility awaits further experimental testing.

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