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ORIGINAL PAPER

Carotenoid-based ornamentation as a dynamic but consistent individual trait

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Abstract Carotenoid-based ornaments act as signals of quality in many animal species. In contrast to feathers, which are relatively stable structures, carotenoid-pigmented integuments (e.g. bills, lores, tarsi) can change colour rapidly and may better reflect changes in physiological condition. I studied the seasonal variations in plasma carotenoids in red-legged partridges (*Alectoris rufa*) kept on a constant diet and free of intestinal parasites. Furthermore, I analyzed whether seasonal changes in circulating carotenoids were mirrored by the carotenoid-based coloration of eye rings and bill of this species. Plasma carotenoids showed seasonal variation, with higher levels coinciding with the end of the mating and the start of the laying season. Eye ring pigmentation was related to plasma carotenoid levels, and changes in bill hue (but not changes in UV or red bill chroma) mirrored the variation in plasma carotenoids during the breeding season. Despite the seasonal variation, individual differences in eye ring pigmentation and bill hue, UV and red chroma were consistent throughout the breeding season. Similarly, individual differences in eye ring pigmentation and bill hue and red chroma remained consistent between consecutive years. These results suggest that carotenoid based integumentary colorations act as dynamic traits that accurately reflect the carotenoid-status of individuals, thus reliably indicating consistent differences in individual quality. Furthermore, variability in signal expression appears to have a relevant genetic/phenotypic basis independently of environmental conditions.

Keywords *Alectoris rufa* · Carotenoids · Sexual selection · Honest signaling · Ultraviolet coloration

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Introduction

Carotenoid pigments are responsible for the yellow-orange-red coloration of many sexual ornaments in animals and they have captured the attention of behavioural ecologists during the last decade as potential signals of individual quality (Olson and Owens 1998). Special interest has been paid to elucidating the proximate basis of variability in carotenoid-based ornament expression. Three main hypotheses have been suggested to explain the variability and the cost of carotenoid ornament expression (a key point for their use as honest signals, Zahavi 1975). These hypotheses are: a) that carotenoids are a scarce resource that is more available for good foragers; b) that carotenoids, apart from pigmentation, have other physiological functions (i.e. antioxidants and immunostimulants) and only high quality individuals (for instance, those free of parasites or diseases) can allocate greater amounts for ornamentation; and c) that carotenoids may be toxic for the individual, and therefore only healthy individuals with prime detoxification systems could manage them for ornament expression (see Olson and Owens 1998; Møller et al. 2000; and references given therein). Current evidence suggest that carotenoid-based ornaments are strongly influenced by both external (i.e. carotenoid availability) and internal (i.e. parasites) environ-

mental factors (e.g. Møller et al. 2000; Hill 2002). However, interindividual variability in carotenoid expression similar to that found in the wild has been observed even when carotenoid access and health status are standardized (e.g. McGraw and Hill 2001; Hadfield and Owens 2006). This suggest that intrinsic physiological or genetic factors affect post-consumption ability to absorb, transport, transform and deposit carotenoids in the ornaments, thus contributing to variability in colour expression.

In birds, carotenoid-based plumage coloration may signal individual quality (i.e. dominance, body condition, parasite levels, breeding success) (review in Hill and McGraw 2006). However, because feathers are non-living structures, their colour is only indicative of a bird's physiological status at the time when feathers were grown. As moult usually takes place several months before the mating-breeding season (except in species with prenuptial moult), there is a time lag between signal production and the time when signals are used in a sexual context. This could potentially reduce the reliability of the signal, as the quality of the bearer may have changed in that period of time, whereas signal expression does not.

In contrast to feathers, fleshy or bare integumentary parts (i.e. cere, tarsus, lores, eye rings, combs, bills) often have the potential to change either colour or shape rapidly (Rosen and Tarvin 2006; Velando et al. 2006). Thus, they could provide reliable, accurate and updated information about current physical condition of an individual (e.g. Faivre et al. 2003; Velando et al. 2006; Martínez-Padilla et al. 2007). In many species, these exposed integumentary parts are brightly coloured by carotenoids (e.g. Negro et al. 1998; Blount et al. 2002; Faivre et al. 2003; McGraw 2004; Velando et al. 2006; Mougeot et al. 2007a). Although recent studies have focused on this kind of traits, more intense research is required to improve our understanding of functional aspects of their use as honest signals.

Levels of circulating carotenoids in plasma usually show seasonal variation (Hill 1992; McGraw and Gergory 2004). Although these variations may be partially attributed to seasonal changes in diet, experiments in captivity showed seasonal variation despite constant diet in american kestrels (*Falco sparverius*) (Negro et al. 1998) and red-legged partridges (*Alectoris rufa*) (Negro et al. 2001), suggesting that other factors apart of environmental availability regulate physiological levels of these pigments. Furthermore, these seasonal patterns of variation seem consistent with sexual selection and signal production. For instance, plasma carotenoid levels are higher during moult in species that show carotenoid-based plumage pigmentation (Hill 1992; McGraw and Gergory 2004). In the case of species with fleshy or unfeathered integumentary carotenoid-based ornaments, such as American kestrels, Negro et al. (1998) found that plasma carotenoids peaked during the mating season and decreased thereafter. Similarly, integuments were more colourful in spring (mating season) than in winter. However, in that study colour and plasma carotenoid sampling were not coupled, plasma carotenoids were measured at four stages during the breeding season but integument coloration was only compared between winter and spring (Negro et al. 1998). Detailed studies where the seasonal change in plasma carotenoids and coloration are simultaneously monitored are still lacking.

The strong environmental determination of carotenoid-based ornaments is at the same time the basis and a critical point for its reliability as honest signals. If the absolute value of a sexual trait (for instance, a plumage ornament) changes from one year to the next (Pärt and Qvanström 1997; Hill 2002), this could potentially confound the receiver about the honesty of the emitter's advertisement. However, traits may still be reliable if they reflect current status or changes in status over the time, and if the relative differences between individuals are maintained over time (Greenfield and Rodríguez 2004; Senar and Quesada 2006). Thus, the most ornamented individuals in the population in one year would continue to be the most ornamented over following years, even if environmental conditions may change the average population value of the trait (Senar and Quesada 2006). This has been shown for plumage traits, which remain reasonably stable between moults. But what about integumentary coloration, which may change strikingly whithin days or hours (Faivre et al. 2003; Rosen and Tarvin 2006; Velando et al. 2006)? The dynamic nature of integument carotenoid-based coloration could compromise the reliability of the signalling system if, in the absence of changes of individual quality, within-individual changes in colour jeopardizes the individual variation in ornamentation (i.e. colour expression relative to others in the population). Despite the capital importance of this issue, it has never been properly analyzed.

In this paper I studied individual variation in plasma carotenoids and carotenoid-based integumentary colour within and between years in captive red-legged partridges fed a constant diet. The red-legged partridge is a medium-sized galliform showing red carotenoid-based coloration (Pérez-Rodríguez and Viñuela, unpublished data) in the exposed integuments of tarsi, bill and eye rings. Sexual dichromatism has been reported for the eye rings, with males showing higher relative area of the eye ring covered by carotenoids (Pérez-Rodríguez and Viñuela, unpublished data), and more intense red coloration in those pigmented areas (Villafuerte and Negro 1998). The aim of this study is to analyze whether birds of both sexes show seasonal variations in plasma carotenoids despite constant diet and whether these variations were associated with changes in carotenoid-based integumentary coloration. Furthermore, I analyze whether carotenoid levels and integumentary

coloration varied consistently among individuals throughout the breeding season and between consecutive years (that is, whether redder birds were consistently redder during the whole breeding season and between consecutive years under controlled environmental conditions for the entire population).

Material and methods

Study design

This study was carried out between 2002 and 2006 in a captive population of red-legged partridges at the facilities of *Dehesa de Galiana (Instituto de Investigación en Recursos Cinegéticos)*. Birds used in the study hatched in the spring of 2002, were reared in communal outdoor pens and fed with a mixture of commercial pelleted food (20% protein, 4.5% fat, 3.7% cellulose; carotenoid content: 5.26 μgr/gr) and wheat. In February 2003, 110 birds (65 males, 55 females) were individually housed in elevated cages (1×0.5×0.4 m) visually isolated from each other, at ambient temperature and natural photoperiod (further details on housing conditions are given in Pérez-Rodríguez et al. 2006). At the time of individual isolation all birds were medicated against coccidia by adding sulfaquinoxaline, a coccidiostatic of common use in partridge farms, to drinking water (1ml/L during one week). The maintenance of birds in elevated cages usually prevents infection by intestinal parasites. In any case, after the preventive treatment described above and throughout the study period, faecal samples from all birds were regularly collected (every 4–5 months, approximately) and faecal analyses confirmed that birds were not infected by coccidia or helminths. There were no ectoparasites in our captive study population. During individual isolation, caged birds were fed only with the pelleted food mentioned above. These housing conditions and diet were maintained unchanged throughout the study period.

Blood samples (aproximately 300 μl) were collected from each bird from the brachial vein using heparinized syringes on the following dates: 28 November 2002, 10 February, 17 March, 8 April, 19 May and 30 October 2003. Although plasma carotenoids do not show significant daytime variation (Pérez-Rodríguez et al. 2007), all blood samples and measurements were obtained approximately at the same hour of the day (between 10:00 and 12:00 hours) in order to avoid any bias due to sampling time. Blood samples were kept cold (4°C) and centrifuged within 8 hours, and plasma was stored at –20°C until carotenoid levels were quantified (no later than two months after sample collection). For the assays, 60 μl of plasma were diluted in acetone (1:10). The mixture was vortexed and centrifuged at 10000 rpm for 10 minutes to precipitate the flocculant proteins; the supernatant was examined in a Shimadzu UV-1603 spectrophotometer and the optical density at 446 nm was determined. Carotenoid concentrations were calculated using a standard curve of lutein (Sigma Chemicals). The carotenoid basis of the red coloration of the eye ring and bill of the red legged partridge was assessed following the method proposed by McGraw et al. (2005).

At the time of each blood sampling (except in November 2002), high resolution (2272×1704 pixels) digital pictures of the left side of the head of each bird were taken under a fluorescent light illumination and against a white standard background to quantify eye ring carotenoid pigmentation (see below).

A preliminary analysis of data obtained during 2002–2003 revealed seasonal variation both in plasma carotenoids and eye ring pigmentation (see Results and Fig. 2). Subsequently, I selected a random subsample (30 males and 23 females) of the birds employed in 2002–2003 to perform a more detailed analysis of plasma carotenoids and colour variation during the following breeding season. The mating period of the species at this latitude ranges from February to late March, whereas the laying period ranges from April to mid June (Cramp and Simmons 1980; author, personal observations). For this purpose, and in order to cover all the breeding season from mating to laying, study birds were sampled as described above at the following dates: 20 January, 3 March, 6 April and 26 May 2004.

Furthermore, in 2004 and in order to obtain a more complete and accurate estimation of colour expression, in addition to scoring eye ring pigmentation from digital pictures, I also measured bill colour with a reflectance spectrometer (see Mougeot et al. 2007a for technical details). The bill was illuminated using a deuterium-halogen light source (DH2000, Top Sensor System) and reflectance values (in 3 nm-steps from 300 to 700 nm) were obtained by using a Spectralon® 99% white standard reference (Labsphere, Congleton). Three measurements (see repeatabilities below) of each bill were done in a partially dark room avoiding the effect of ambient light.

To test for the consistency of plasma carotenoid levels and carotenoid-based coloration between consecutive years, I collected blood samples, digital pictures and spectrometric measurements of some of these males in 2005 (7 April) and 2006 (4 April). This was done only for a subsample of males (21 in 2005 and 19 in 2006), but not for females. Not all variables were measured in all study birds, so sample sizes vary.

Eye ring colour scoring

The eye ring of the red-legged partridge shows a striking degree of variation in the amount of bare skin around the

eye pigmented by carotenoids or unpigmented (i.e. showing the white-underlying dermis). In order to capture this variability, that otherwise would be not quantified by most commonly used methods to measure coloration, I defined a five-level colour scale attending to the relative area of bare skin covered by red pigment: 1- red pigment almost completely absent, restricted to the edges of the eyelid and with none or very few small spot of red pigment in the bare white skin around the eye; 2- red pigment covering 50% or less of the eye ring, large amount of white skin visible; 3- greater amount of bare area covered by red pigment, but some continuous areas of white skin still visible; 4- almost all area covered by red pigment, only small spots of white skin visible; and 5-bare skin and eye ring completely covered by red pigment (see Electronic Supplementary Material S1 for illustrative examples and further details on eye ring pigmentation scoring).

Bill colour variables

Bills are not flat surfaces. Therefore, minimal changes in the angle of the reflectance probe may cause small changes in the spectral curve, which can be transposed up or down while retaining the same shape (Grill and Moore 1998). This may affect colour chroma as it is a relative measure referred to the total reflectance (i.e. brightness). Therefore, all spectra were standardized to a common area under the curve (by dividing reflectance at each wavelength by total brightness) before calculating hue and chroma (Endler 1990; Grill and Moore 1998). In addition to reflecting light maximally at red wavelengths, the bill of the red-legged partridge shows a lower reflectance peak in the UV (Fig. 1). As most of birds can see in the UV wavelength range (Hill and McGraw 2006), this was also included in the study. Therefore, I calculated UV-chroma (total reflectance in the 300–400 nm interval, in percent, relative to total brightness), red chroma (total reflectance in the 600–700 nm interval, in percent, relative to total brightness), UV hue (wavelength of maximum reflectance in the 300–400 nm interval) and red hue (wavelength of maximum slope) (Endler 1990, Grill and Moore 1998). In a preliminary analysis of a subsample of birds (N=64) UV hue showed a very low repeatability (after Lessells and Boag 1987; r = 0.30, $F_{67,136}$ = 2.3, P<0.001). Therefore I excluded this variable from the study. Repeatabilities of the other three colour variables were high (UV chroma: r = 0.77, $F_{163,328}$ = 11.5, P<0.001; red chroma: r = 0.81, $F_{163,328}$ = 14.1, P<0.001; red hue: r = 0.88, $F_{163,328}$ = 23.6, P<0.001).

Statistical analyses

Although data from 2002–2003 were collected from the same pool of 110 birds, not all birds were sampled at each sampling event. Therefore, a simple (non-repeated) two-way ANOVA with sex, month and their interaction as fixed factors was performed to analyze seasonal variations in plasma carotenoids and bill colour variables (all of them met the assumptions of normality and homoscedasticity). In the case of eye ring colour score, a Generalized Linear Model (GLZ), assuming multinomial ordinal distribution and logit link function, was employed.

In 2004, I followed a repeated-measures design. Therefore, to analyse the effect of sex and carotenoid levels on seasonal (January to May) variation in bill colour variables, I used General Linear Mixed Models (MIXED procedure and REPEATED statement from SAS software, Littell et al. 1998). Each colour variable was entered as the dependent variable, whereas sex, month and plasma carotenoid levels, as well as all double interactions, were entered as fixed variables. Individual identity was included as a random factor. Non-significant terms were sequentially excluded from the model using a backward stepwise selection. For eye ring pigmentation, a GLZ (multinomial ordinal distribution and logit link function) with the same fixed variables

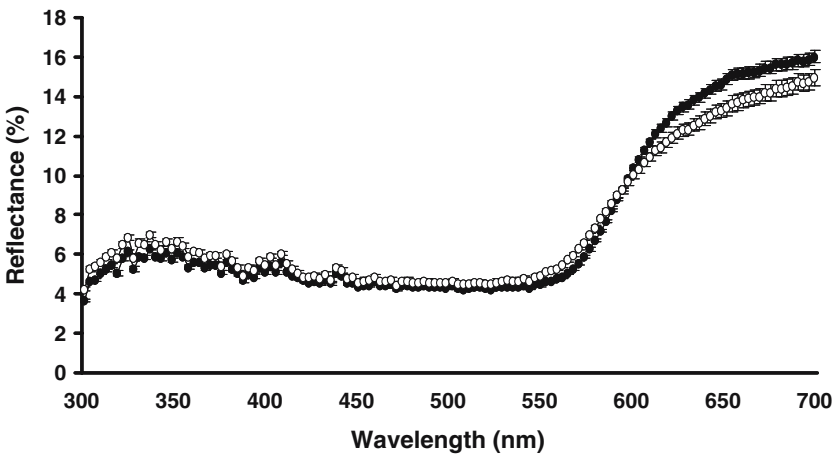

Fig. 1 Average (±SE) reflectance curves in the 300–700 nm interval of male (closed circles) and female (open circles) red-legged partridge bills during the breeding season (January-May) of 2004

and interactions was performed. In the case of plasma carotenoids, I used the same model, but entering only sex, month and their interaction.

For the 2004 data, I computed inter-month correlations in plasma carotenoids and eye and bill colour variables using a GLM for each variable. The variable "1st month" was entered as an independent factor, whereas the value of the variable measured in the following month was entered as a covariate. The interaction between "1st month" and the covariate was also entered to test for differences in slope between months. As sexual differences were found in colour variables and in their seasonal variations (see Results), sexes were analyzed separately. A similar statistical approach was used to analyze inter-year correlation, but entering "1st year" as a factor, with the value of the analyzed variable in April of the first year and its value in April of the following year as covariate and response variable respectively. I selected this month for inter-year comparisons because it is the period when plasma carotenoids and bill hue reached their maximum values (see Fig. 3a,b)

Results

Seasonal variation in plasma carotenoids and carotenoid-based coloration

Plasma carotenoids showed significant seasonal variation from November 2002 to October 2003 ($F_{6,361}$ = 80.8, P<0.001), with higher levels during the mating-breeding season (March to June) than during the rest of the year (Fig. 2). There was no overall effect of sex nor sex × month interaction ($F_{1,361}$ = 1.21, P=0.27 and $F_{6,361}$ = 1.14, P=0.33). Eye ring pigmentation showed similar seasonal variation (Wald = 20.0, df = 5, P<0.01), but males showed higher eye ring carotenoid pigmentation than females (Wald = 32.6, df = 1, P<0.001) (Fig. 2).

In 2004, I tested whether a significant change in bill colour and eye ring pigmentation existed during the mating-breeding season, and whether this variation differed between sexes and was linked to changes in plasma carotenoid levels. Results are shown in Table 1 and Fig. 3. Plasma carotenoids varied significantly from January to late May. Males had higher levels in April-May, when plasma carotenoids sharply decreased in females but not so much in males (significant sex × month interaction, Table 1).

Eye ring pigmentation did not show significant monthly variation in either sex, although eye ring pigmentation tended to increase in late May in males but not in females. These variations were significantly associated to variation in plasma carotenoids and the eye ring of males was overall more pigmented than that of females (Table 1, Fig. 3).

Bill UV and red chroma followed parallel but totally opposed monthly and sexual patterns of variation (Fig. 3). Male's bills had higher red chroma and lower UV chroma than those of females, especially during January-March. Furthermore, monthly variation differed between sexes. The decrease in UV chroma and increase in red chroma from January to April was more pronounced in females than males (Fig. 3). However, these changes were not related to variation in circulating carotenoids (Table 1). In contrast, bill hue variation was strongly associated to plasma carotenoid changes from January to late May, as illustrated by the similarity of their monthly patterns (Fig. 3). Males showed higher bill hues (redder bills) than females, the later showing a sharp decrease in bill hue from April to late May, as found for plasma carotenoids.

Despite being individually isolated, in 2004 (but not in 2003, probably because they were in their first breeding season) 77% of our study females laid eggs in the interval between April and May samplings (average number of eggs laid among layers: 4.5±2.6, range 1–9). However, I did not find significant correlations between the number of eggs

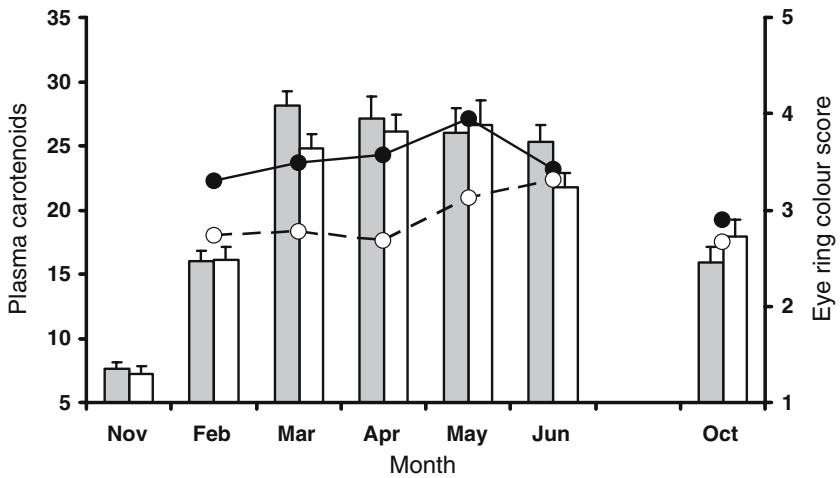

Fig. 2 Variation in plasma carotenoids (columns, left axis) and eye ring pigmentation score (lines, right axis) in red-legged partridges from November 2002 to October 2003. Grey columns, black circles and solid line correspond to males and open columns and circles and dashed lines to females. Error bars indicate SE, and have been omitted in colour scores for clarity

Table 1 Results of the models analyzing the variation in plasma carotenoids and carotenoid based coloration in red-legged partridges during the breeding season

	Plasma		Eye ring		Bill					
	Carotenoids		Colour score		UV chroma		Red chroma		hue	
	F (*df*)	*P* value	Wald (*df*)	*P* value	*F* (*df*)	*P* value	*F* (*df*)	*P* value	*F* (*df*)	*P* value
Month	22.8 (3,100)	<0.001	2.81 (3)	0.41	17.9 (3,117)	<0.001	6.35 (3,117)	<0.001	3.69 (3,92)	0.01
Sex	8.98 (1,41)	<0.01	4.47 (1)	0.03	9.72 (1,79)	<0.01	7.38 (1,79)	<0.01	7.97 (1.36)	<0.01
Plasma carotenoids	-	-	6.13 (1)	0.01	1.62 (1,95)	0.20	0.15 (1,95)	0.69	15.4 (1,92)	<0.001
Month × sex	10.61 (3,100)	<0.001	0.88 (3)	0.83	2.85 (3,117)	0.04	3.05 (3,117)	0.03	4.06 (3,92)	<0.01
Month × plasma carotenoids	-	-	2.03 (3)	0.56	0.09 (3,91)	0.96	0.27 (3, 91)	0.85	0.77 (3,88)	0.51
Sex × plasma carotenoids	-	-	0.15 (1)	0.69	0.26 (1,94)	0.61	0.29 (1,94)	0.59	1.21 (1,91	0.27

For plasma carotenoids and bill colour variables, GLMs were implemented with individual entered as a random factor and each month (January, March, April and May) as the different levels of the repeated factor. Sex and plasma carotenoids (only for colour variables) were entered as fixed variables. For eye ring pigmentation a GLZ with the same fixed variables was performed. Also given are statistics and p-values of non significant terms at the time when they were excluded from the final model.

laid and the change from April to May in any of the parameters studied (plasma carotenoids, eye ring pigmentation or bill colour variables, all $P>0.24$). Results were similar when we compared the change in these variables between females that laid vs. females that did not lay any egg in this interval (all $P>0.31$), except in the case of bill hue, that showed lower changes from April to May in females that did not lay any egg (ANOVA, $F_{1,14} = 5.59$, $P=0.03$).

Inter-month and inter-year relationship in plasma carotenoids and carotenoid-based coloration

Within-individual plasma carotenoid levels were not correlated between consecutive months ($F_{1,47} = 1.43$, $P=0.23$ and $F_{1,45} = 0.45$, $P=0.46$, in males and females, respectively) (Fig. 4). In contrast, I found significant positive relationships for eye ring pigmentation (males: Wald = 20.0, $df = 1$, $P<0.001$; females: Wald = 29.4, $df = 1$, $P<0.001$), bill UV chroma (males: $F_{1,62} = 24.0$, $P<0.001$; females: $F_{1,46} = 4.28$, $P=0.04$), bill red chroma (males: $F_{1,62} = 20.8$, $P<0.001$; females: $F_{1,46} = 4.82$, $P=0.03$) and bill hue (males: $F_{1,61} = 32.6$, $P<0.001$; females: $F_{1,42} = 17.2$, $P<0.001$) (Fig. 4). Interactions between month and the covariate were non significant in all cases (all $P>0.30$).

Similarly, I did not find significant relationships between April values of consecutive years either in plasma carotenoid levels ($F_{1,21} = 0.48$, $P=0.49$) or in bill UV chroma ($F_{1,11} = 2.39$, $P=0.15$) (Fig. 5). In contrast, relationships were significant for eye ring pigmentation (Wald =12.36, $df = 1$, $P<0.001$), bill red chroma ($F_{1,11} = 6.58$, $P=0.02$) and bill hue ($F_{1,11} = 10.2$, $P<0.01$) (Fig. 5). Interactions between years and the covariate were non significant in all cases (all $P>0.15$).

Discussion

In this study I found that red-legged partridges show seasonal variation in plasma carotenoid levels despite a constant diet, and that these changes were reflected by changes in carotenoid-based coloration of the eye rings and bills. Interestingly, besides this dynamism in colour expression, red colour expression relative to others in the study population was consistent across the breeding season and between consecutive years.

Although seasonal changes in plasma carotenoid levels may be attributed to changes in the diet of wild birds, studies on captive birds where diet was held constant also detected seasonal changes in plasma carotenoids (Negro et al 1998, 2001; this study), suggesting that internal factors may regulate carotenoid absorption, transport or mobilization. I found that plasma carotenoids and eye ring pigmentation in red-legged partridges were higher during the breeding season (from March to June) in one year-old birds that were kept under constant diet. Similarly, in two year-old birds, plasma carotenoids peaked in March-April, coinciding approximately with the end of the mating season of the species and the start of egg laying (Cramp and Simmons 1980; author, personal observation). In contrast to this, Negro et al. (2001) found that plasma carotenoid levels in this species peaked in late February, strongly decreased in early April and increased again in May. However, as discussed by the authors, the captive population of Negro et al. suffered a coccidiosis outbreak during the study, which may have affected carotenoid absorption (Allen 1992), a possibility discarded in this study since gut parasite levels were controlled.

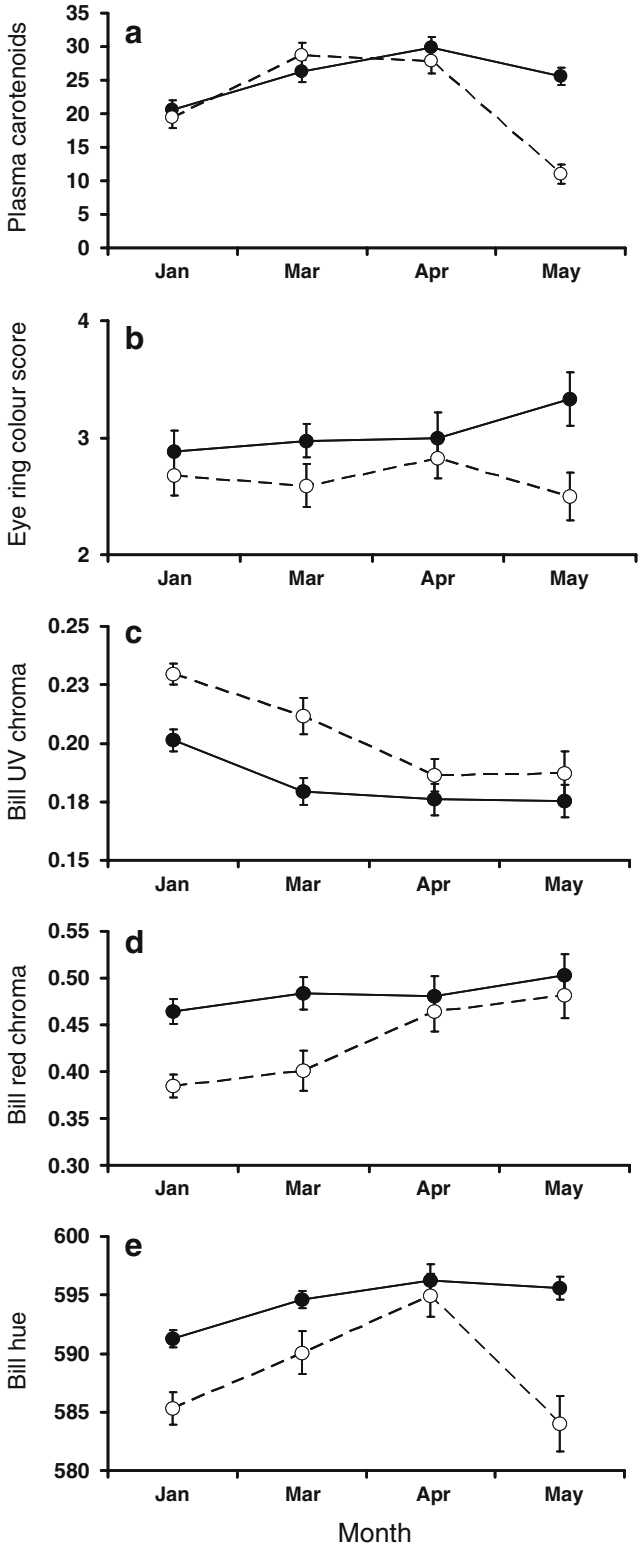

Fig. 3 Variation in plasma carotenoids and carotenoid-based coloration during the breeding season of 2004. **a** plasma carotenoid levels (μg/mL), **b** eye ring colour score, **c** bill UV chroma, **d** bill red chroma, and d) bill hue. In all cases, closed circles and solid lines correspond to males whereas open circles and dashed lines correspond to females. Values are means±SE

In addition to plasma carotenoids, carotenoid-based bill coloration also changed during the breeding season (from January to late May). In fact, the seasonal pattern of variation of bill hue exactly mirrored that of plasma carotenoids (Fig. 3), suggesting that this colour variable accurately reflects the circulating levels of these pigments. Furthermore, although eye ring pigmentation did not change significantly during the breeding season, it was significantly associated with circulating levels of carotenoids from January to late May. The fact that plasma carotenoids and carotenoid-based ornaments were maximal during the breeding season and showed sexual dimorphism is consistent with a role in sexual selection (Negro et al. 1998). In this species, the mating period ranges from February to late March. However, most colour traits peaked later (in April or May). A possible explanation for this delay is that the egg laying period extends up to mid June, and therefore males may have been selected to maintain signalling effort to stimulate mate investment and fidelity. Although the role of carotenoid-based ornaments on sexual selection in this species has not been experimentally assessed, there are many examples of similar traits in other species whose signalling function is well known (e.g. Omland 1996; Rintamaki et al. 2000; Bright and Waas 2002; Blount et al. 2003). Furthermore, there is assortative mating with respect to carotenoid-based coloration in this species in the wild (F Mougeot, F Casas and L Pérez-Rodríguez, unpublished data). This, together with the sexual dimorphism (Villafuerte and Negro 1998; this study) and the condition-dependence of these traits (Pérez-Rodríguez and Viñuela, unpublished data), is indirect support of the importance of these traits in the sexual selection process of the red-legged partridge.

Recent studies have reported an association between testosterone and plasma carotenoids (McGraw et al. 2006; Blas et al. 2006). Peak values of testosterone in male partridges are reached in April (Bottoni et al. 1993), which may explain the seasonal pattern in plasma carotenoids and carotenoid-based coloration described here. The explanation is valid for both sexes, as the seasonal variation of this hormone is similar in females too (Carter 1992). Furthermore, in 2004 females showed a drop in plasma carotenoids and bill hue from April to May. This may be explained by carotenoid allocation to egg yolk formation, which has been shown to affect plasma carotenoids in this species (Bortolotti et al. 2003). However the overall association between egg laying and the degree of change in colour or plasma carotenoids was weaker than previously reported (Bortolotti et al. 2003), probably because in this study females were individually housed and may have allocated less carotenoids to eggs with no probabilities of being fertilized. Possible effects of the age of reproductive females or the total number of eggs laid (much greater in the study of Bortolotti et al. 2003) may also explain these apparently contrasted results.

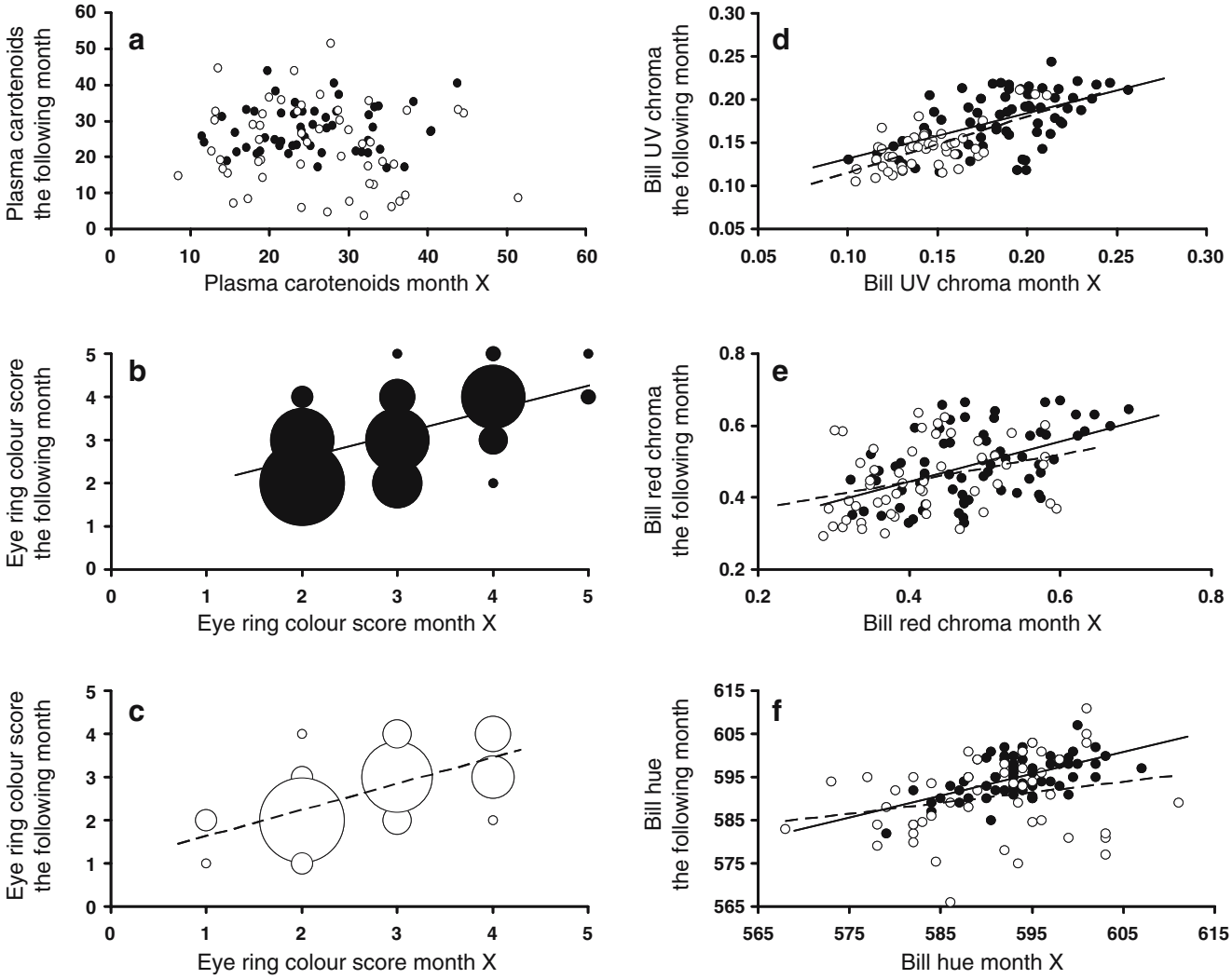

Fig. 4 Relationship between subsequent samplings in the values of plasma carotenoids and carotenoid-based coloration during the breeding season of 2004. **a** plasma carotenoid levels (μg/mL), **b** eye ring colour score in males, **c** eye ring colour score in females, **d** bill UV chroma, **e** bill red chroma, and **f** bill hue. In all cases, closed circles and solid regression lines (added only when a significant relationship was detected) correspond to males whereas open circles and dashed regression lines correspond to females. Circle size is proportional to the number of overlapping points

Although the enhancement of carotenoid-based traits during the mating season appears to be the most reasonable explanation for the seasonal pattern found, we cannot exclude some other possibilities. For instance, this variation may be attributed to a decreasing physiological demand of carotenoids for other activities in spring-summer, or to an increase in food intake. However, the progress of the breeding season is associated with an increase of testosterone and metabolic rate, which may result in higher oxidative stress (Alonso-Alvarez et al. 2007). As carotenoids are part of the antioxidant machinery of the individual, an increasing (instead of a decreasing) demand of carotenoids is expected. In addition, in this captive population food intake tends to decrease in spring (Blas et al. 2006) and therefore the seasonal pattern found here is not consistent with this change in food/carotenoid intake. Furthermore, inter-individual differences in plasma carotenoids are not explained by daily food intake (Pérez-Rodríguez and Viñuela, unpublished data). Finally, no ectoparasites were detected in our study population, birds were medicated against intestinal parasites and they showed no apparent signs of disease (such as weight loss or reduced activity) during the study. We cannot exclude a possible effect of seasonal variations in other pathogens (such as blood parasites) that may exert a negative effect on plasma carotenoids and ornamentation (e.g. Hõrak et al. 2001). However, blood parasites are expected to show higher abundances during the breeding season (Valkiunas 2005), which is not consistent with the pattern described here.

In contrast to red bill hue, variation in bill UV and red chroma was not related to changes in plasma carotenoids during the breeding season. In fact, these two variables showed patterns that were totally opposed: while red

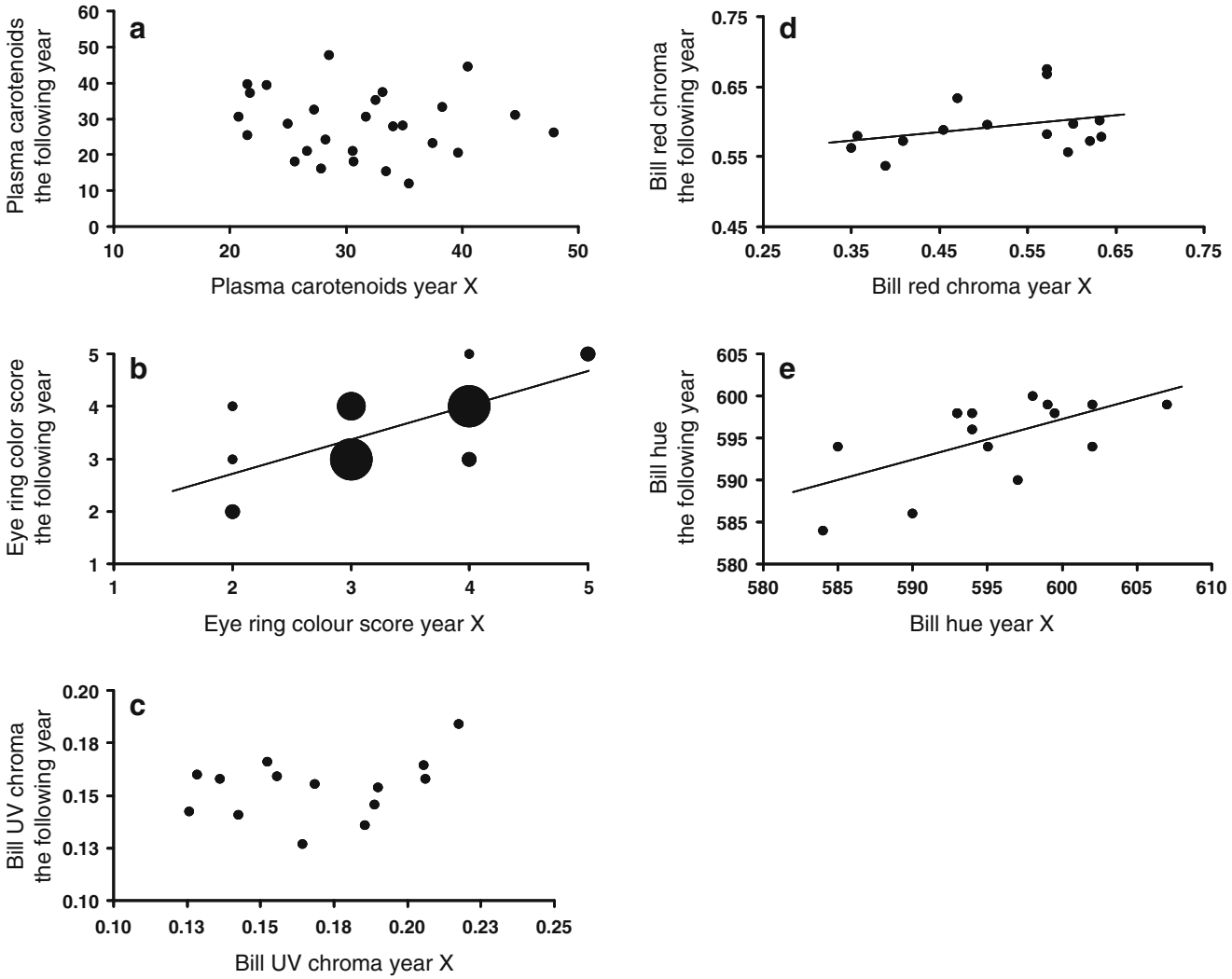

Fig. 5 Relationship between values of plasma carotenoids and carotenoid based coloration of male red-legged partridges in April of subsequent years. **a** plasma carotenoid levels (μg/mL), **b** eye ring colour score, **c** bill UV chroma, **d** bill red chroma, and **e** bill hue. Regression line has been added only when significant relationships were detected. Circle size is proportional to the number of overlapping points

chroma increased from January to May, UV chroma decreased. Furthermore, males showed on average higher red chroma and lower UV chroma (and higher hues) than females. Dresp et al. (2005) showed that the UV reflectance by the bill of king penguin (*Aptenodytes patagonicus*) was caused by the microstructure of the external layers of the bill. If the same mechanism applies to the red-legged partridge, it is possible that UV reflectance of the outer (and probably static) layers of the bill remained constant throughout the breeding season, whereas the carotenoid-based pigmentation of inner (metabolically active) bill layers increased. As chroma is calculated as reflectance value relative to the total reflectance in the whole spectrum, the increased reflectance at red wavelengths (600–700 nm) might result, as a side-effect, in lower relative values in the UV (300–400 nm) interval (lower UV chroma), explaining the opposed pattern of red and UV.

It is intriguing that plasma carotenoid variation was associated with some bill colour features (i.e. hue) but not others (UV and red chroma). In plumage traits, both feather hue and chroma reflect its total carotenoid content (Saks et al. 2003) and are affected by changes in the relative concentration of each carotenoid type in the feather (McGraw et al. 2004). Future studies should assess the relationship between colour features and the relative proportion, as well as the total amount, of the different carotenoids of the bill and eye rings in the red-legged partridge and whether seasonal changes on carotenoid integumentary composition explain the observed changes on each colour feature.

An important finding of this study is that, despite seasonal variability, there was a consistency in eye ring pigmentation and bill colour within individuals. Therefore, colour expression relative to others in the study population was maintained during the whole breeding season in both

sexes, with redder individuals remaining the redder ones from January to May. Similarly, Dawson and Bortolotti (2006) found correlated expression of carotenoid-based integumentary coloration at two different phases of the reproductive season (prelaying and incubation) in american kestrels. Furthermore, I found similar results when data from consecutive years were compared, as those individuals with higher eye ring pigmentation and bill red chroma and hue remained so the following year. This study is the first to show that carotenoid-based integumentary coloration is a dynamic trait that remains consistent between individuals (within and between years). Regarding carotenoid-based plumage traits, a similar inter-year correlation was found by Senar and Quesada (2006) in wild great tits (*Parus major*). However, in contrast to colour variables, I did not find a similar consistence in plasma carotenoid levels, neither at the intra-seasonal nor inter-annual levels. This may be attributed to the fact that plasma carotenoids levels show important short term variations (due, for instance, to time elapsed since last food intake) that may obscure any general pattern.

It should be noted that colour expression relative to others in the population was maintained within the breading season and between years when all birds had *ad libitum* access to the same diet and after removal of intestinal parasites. Therefore, we experimentally controlled for most of the environmental factors known to affect carotenoids (Olson and Owens 1998, Møller et al. 2000). This implies that inter-individual variability in colour expression was mainly attributable to internal factors. Furthermore, since all birds remained unpaired and in the same housing conditions during the study, differences in signalling effort between individuals due to variability in previous mating success or future survival or reproduction were not expected (Badyaev and Duckworth 2003). Therefore, colour expression may reflect the capacity of an individual to absorb, transport and metabolize the ingested carotenoids and deposit them in ornaments. As some of these processes may be linked to testosterone in both sexes (McGraw 2006; McGraw et al. 2006; Blas et al. 2006; Mougeot et al. 2007b), inter-individual variability in the capacity to bear the costs of increased testosterone levels may be another source of variation of colour expression. In this sense, a dynamic signal such as carotenoid-based integumentary coloration could not only provide information about physiological status of the individual (e.g. nutritional status, parasite levels), but also about its quality independently of environmental conditions. Future studies should target these sources of individual variability of colour expression when environmental factors are controlled for.

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References

Allen PC (1992) Effect of coccidiosis on the distribution of dietary lutein in the chick. Poult Sci 71:1457–1463

Alonso-Alvarez C, Bertrand S, Faivre B, Chastel O, Sorci G (2007) Testosterone and oxidative stress: the oxidation handicap hypothesis. Proc R Soc Lond B Biol Sci 274:819–825

Badyaev AV, Duckworth RA (2003) Context-dependent sexual advertisement: plasticity in development of sexual ornamentation throughout the lifetime of a passerine bird. J Evol Biol 16:1065–1076

Blas J, Pérez-Rodríguez L, Bortolotti GR, Viñuela J, Marchant TA (2006) Testosterone increases bioavailability of carotenoids: new insights into the honesty of sexual signaling. Proc Natl Acad Sci USA 103:18633–18637

Blount JD, Surai PF, Nager RG, Houston DC, Møller AP, Trewby ML, Kennedy MW (2002) Carotenoids and egg quality in the lesser black-backed gull *Larus fuscus*: a supplemental feeding study of maternal effects. Proc R Soc Lond B Biol Sci 269:29–36

Blount JD, Metcalfe NB, Birkhead TR, Surai PF (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. Science 300:125–127

Bortolotti GR, Negro JJ, Surai PF, Prieto P (2003) Carotenoids in eggs and plasma of red-legged partridges: effects of diet and reproductive output. Physiol Biochem Zool 76:367–374

Bottoni L, Massa R, Lea RW, Sharp PJ (1993) Mate choice and reproductive success in the red-legged partridge (*Alectoris rufa*). Horm Behav 27:308–317

Bright A, Waas JR (2002) Effects of bill pigmentation and UV reflectance during territory establishment in blackbirds. Anim Behav 64:207–213

Carter CS (1992) Neuroendocrinology sexual behaviour in the female. In: Becker JB, Breedlove SM, Crews D (eds) Behavioral. A Bradford Book. MIT Press, Cambridge, pp 71–96

Cramp S, Simmons KEL (1980) The Birds of the Western Paleartic. Oxford University Press, Oxford

Dawson RD, Bortolotti GR (2006) Carotenoid-dependent coloration of male American kestrels predicts ability to reduce parasitic infections. Naturwissenschaften 93:597–602

Dresp B, Jouventin P, Langley K (2005) Ultraviolet reflecting photonic microstructures in the King Penguin beak. Biol Lett 1:310–313

Endler JA (1990) On the measurement and classification of colour in studies of animal colour patterns. Biol J Linn Soc 41:315–352

Faivre B, Grégoire A, Préault M, Cézilly F, Sorci G (2003) Immune activation rapidly mirrored in a secondary sexual trait. Science 300:103

Geenfield MD, Rodríguez RL (2004) Genotype-environmnet interaction and the reliability of mating signal. Anim Behav 68:1461–1468

Grill CP, Moore AJ (1998) Effects of larval antipredator response and larval diet on adult phenotype in an aposematic ladybird beetle. Oecologia 114:274–282

Hadfield JD, Owens IPF (2006) Strong environmental determination of a carotenoid-based plumage trait is not mediated by carotenoid availability. J Evol Biol 19:1104–1114

Hill GE (1992) Proximate basis of variation in carotenoid pigmentation in male house finches. Auk 109:1–12

Hill GE (2002) A red bird in a brown bag. Oxford University Press, Oxford

Hill GE, McGraw KJ (eds) (2006) In: Bird coloration, vol. 2, Function. and evolution. Harvard University Press, Cambridge

Hõrak P, Ots I, Vellau H, Spottiswoode C, Møller AP (2001) Carotenoid-based plumage coloration reflects hemoparasite infection and local survival in breeding great tits. Oecologia 126:166–176

Lessells CM, Boag PT (1987) Unrepeatable repeatabilities: a common mistake. Auk 104:116–121

Littell RC, Henry PR, Ammerman CB (1998) Statistical analysis of repeated measures data using SAS procedures. J Anim Sci 76:1216–1231

Martínez-Padilla J, Mougeot F, Pérez-Rodríguez L, Bortolotti GR (2007) Nematode parasites reduce carotenoid-based signalling in male red grouse. Biol Lett 3:161–164

McGraw KJ (2004) Colourful songbirds metabolize carotnoids at the integument. J of Avian Biol 35:471–476

McGraw KJ (2006) Sex steroid dependence of carotenoid-based coloration in female zebra finches. Physiol Behav 88:347–352

McGraw KJ, Hill GE (2001) Carotenoid acces and intraspecific variation in plumage pigmentation in American goldfinches (*Carduelis tristis*) and Northern cardinals (*Cardinalis cardinalis*). Funct Ecol 15(15):732–739

McGraw KJ, Gergory AJ (2004) Carotenoid pigments in American goldfinches: what is the optimal biochemical strategy for becoming colourful? Biol J Linn Soc 83:273–280

McGraw KJ, Hill GE, Navara KJ, Parker RS (2004) Differential accumulation and pigmenting ability of dietary carotenoids in colourful finches. Physiol Biochem Zool 77:484–491

McGraw KJ, Hudon J, Hill GE, Parker RS (2005) A simple and inexpensive chemical test for behavioral ecologists to determine the presence of carotenoid pigments on animal tissues. Behav Ecol Sociobiol 57:391–397

McGraw KJ, Correa SM, Adkins-Regan E (2006) Testosterone upregulates lipoprotein status to control sexual attractiveness in a colourful songbird. Behav Ecol Sociobiol 60:117–122

Møller AP, Biard C, Blount JD, Houston DC, Ninni P, Saino N, Surai PF (2000) Carotenoid-dependent signals: indicators of foraging efficiency, immunocompetence or detoxification ability? Avian Poultry Sci Rev 11:137–159

Mougeot F, Martínez-Padilla J, Pérez-Rodríguez L, Bortolotti GR (2007a) Carotenoid-based coloration and ultraviolet reflectance of the sexual ornaments of grouse. Behav Ecol Sociobiol 61:741–751

Mougeot F, Pérez-Rodríguez L, Martínez-Padilla J, Leckie F, Redpath SM (2007b) Parasites, testosterone and honest carotenoid-based signaling of health. Funct Ecol. 21:886–898

Negro JJ, Bortolotti GR, Tella JL, Fernie KJ, Bird DM (1998) Regulation of integumentary colour and plasma carotenoids in American Kestrels consistent with sexual selection theory. Funct Ecol 12:307–312

Negro JJ, Tella JL, Hiraldo F, Bortolotti GR, Prieto P (2001) Sex- and age-related variation in plasma carotenoids despite a constant diet in the red-legged partridge *Alectoris rufa*. Ardea 89:275–280

Olson VA, Owens IPF (1998) Costly sexual signals: are carotenoids rare, risky or required? Trend Ecol Evol 13:510–514

Omland KE (1996) Female mallard male preferences for multiple male preferences. II Experimental variation. Behav Ecol Sociobiol 39:361–366

Pärt T, Qvanström A (1997) Badge size in collared flycatchers predict the outcome of male competition over territories. Anim Behav 54:893–899

Pérez-Rodríguez L, Blas J, Viñuela J, Marchant TA, Bortolotti GR (2006) Condition and androgen levels: are condition-dependent and androgen-mediated traits two sides of the same coin? Anim Behav 72:97–103

Pérez-Rodríguez L, Alonso-Álvarez C, Viñuela J (2007) Repeated sampling but not sampling hour affects plasma carotenoid levels. Physiol Biochem Zool 80:250–254

Rintamaki PT, Hoglund J, Karvonen E, Alatalo RV, Bjorklund N, Lundberg A, Ratti O, Vouti J (2000) Combs and sexual selection in black grouse (*Tetrao tetrix*). Behav Ecol 11:465–471

Rosen RF, Tarvin KA (2006) Sexual signals of the male American goldfinch. Ethology 108:1008–1019

Saks L, McGraw KJ, Hõrak P (2003) How feather color reflects its carotenoid content. Funct Ecol 17:555–561

Senar JC, Quesada J (2006) Absolute and relative signal: a comparison between melanin- and carotenoid-based patches. Behaviour 143:589–595

Valkiunas G (2005) Avian malaria parasites and other haemosporidia. CRC Press, Florida

Velando A, Beamonte-Barrientos R, Torres R (2006) Pigment-based skin colour in the blue-footed booby: an honest signal of current condition used by females to adjust reproductive investment. Oecologia 149:135–142

Villafuerte R, Negro JJ (1998) Digital imaging for colour measurement in ecological research. Ecol Lett 1:151–154

Zahavi A (1975) Mate selection- a selection for a handicap. J Theor Biol 53:205–214