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RESEARCH ARTICLE

Dietary canthaxanthin reduces xanthophyll uptake and red coloration in adult red-legged partridges

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ABSTRACT

Carotenoids give color to conspicuous animal signals that are often the product of sexual selection. Knowledge of the mechanisms involved in carotenoid-based signaling is critical to understanding how these traits evolve. However, these mechanisms remain only partially understood. Carotenoids are usually viewed as scarce dietary antioxidants whose allocation to ornaments may trade off against health. This trade-off would ensure its reliability as a signal of individual quality. In the case of red (keto)carotenoids, the literature suggests that some species may show constraints in their uptake. Canthaxanthin is one of the most common ketocarotenoids in red ornaments of animals. It is often commercially used as a dietary supplement to obtain redder birds (e.g. poultry). We increased the dietary canthaxanthin levels in captive red-legged partridges (*Alectoris rufa*). This species shows red non-feathered parts mostly pigmented by another common ketocarotenoid: astaxanthin. We studied the impact on the uptake of carotenoids and vitamins and, finally, on coloration. We also tested the potential protective effect of canthaxanthin when exposing birds to a free radical generator (diquat). Canthaxanthin did not apparently protect birds from oxidative stress, but interfered with the absorption of yellow carotenoids (lutein and zeaxanthin). Zeaxanthin is a precursor of astaxanthin in enzymatic pathways, and their levels in tissues and eggs were lower in canthaxanthin-supplied birds. This led to lower astaxanthin levels in ornaments and paler coloration. As far as we know, this is the first report of a carotenoid supplementation decreasing animal coloration. The results have implications for understanding carotenoid-based signaling evolution, but also for improving husbandry/experimental procedures.

KEY WORDS: Competitive carotenoid absorption, Dietary pigments, Gallinacean, Poultry diet, Sexual selection, Sexual signals

INTRODUCTION

A plethora of natural carotenoid molecules (about 700) have been found and described in living beings, including bacteria, plants and other organisms (Goodwin, 1984; Britton et al., 2009). They are antioxidants with pigmentary properties (Britton, et al., 2009). In vertebrates, some carotenoids produce conspicuous yellow-to-red coloration that is involved in communication (e.g. colorful plumage in birds and skin patches of reptiles, amphibians and fishes). Most of these traits would have evolved under sexual selection to signal individual quality during intra-sexual competition or mate choice (e.g. Senar, 2006; Hill, 2006).

In spite of the large number of different carotenoids giving rise to animal coloration, not many are involved in the production of red ornaments. Thus, for instance, astaxanthin and/or canthaxanthin are present in 90% of the red traits of avian species (reviewed in table 5.1 in McGraw, 2006). In aquatic birds, these two ketocarotenoids can be directly obtained from the diet because they often feed on invertebrates that store large amounts of these red compounds (Hudon and Brush, 1990; Negro et al., 2000; Matsuno, 2001). In terrestrial birds, however, red ketocarotenoids are mostly obtained by transforming (e.g. oxidizing) yellow xanthophylls abundant in the diet (mainly zeaxanthin and lutein obtained from plants or caterpillars; McGraw, 2006; Isaksson, 2009; García-de Blas et al., 2016). It may be argued that this mechanism could have evolved as a result of a scarcity of ketocarotenoids in the diet of terrestrial birds compared with aquatic birds. However, a recently described enzyme (a ketolase) responsible for creating red ketocarotenoids from yellow xanthophylls (i.e. CYP2J19; see Lopes et al., 2016; Mundy et al., 2016) is also present in red integuments of aquatic turtles (i.e. Twyman et al., 2016), which should *a priori* have better access to dietary red ketocarotenoids, as for aquatic birds. The appearance of carotenoid biotransformation pathways allowing the production of red ketocarotenoid-based colorations could, nonetheless, also have occurred as a result of constraints in ketocarotenoid absorption from the diet, at least in some species.

Large differences among animal species in the capacity to absorb specific carotenoids are revealed by the literature. For instance, in iguanas (*Iguana iguana*) and some birds such as zebra finches (*Taeniopygia guttata*) or canaries (*Serinus canaria*), dietary beta-carotene is apparently poorly absorbed, whereas in other species (particularly aquatic birds), large amounts are accumulated in body tissues (Raila et al., 2002; McGraw, 2006). Differences between taxa in how specific dietary carotenoids compete during intestinal absorption are also common. In humans, high dietary beta-carotene levels inhibit the absorption of yellow carotenoids such as lutein (e.g. Micozzi et al., 1992; Kostic et al., 1995; Brown et al., 1997), but also red ketocarotenoids, including canthaxanthin (e.g. White et al., 1994; Paetau et al., 1997; Salter-Venzon et al., 2017). Dietary canthaxanthin, in contrast, inhibits the uptake of astaxanthin in sea urchins (*Anthocidaris crassispina*; Choubert, 2010) and lycopene in rats (Brown et al., 1997), although it may also increase lutein absorption in iguanas (Raila et al., 2002). Different competitive patterns between the two cited red ketocarotenoids have also been described in closely related species such as rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*); astaxanthin outcompetes canthaxanthin in rainbow trout, whereas the opposite is found in salmon (Kiessling et al., 2003, and references therein). Nonetheless, the impact of this interference on

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the expression of red ketocarotenoid-based ornaments has not been assessed in any of the cited studies.

We recently performed a study manipulating the dietary carotenoids of captive red-legged partridges (*Alectoris rufa*) that reveals strong interactions between carotenoids during absorption and the effect of this on red coloration (García-de Blas et al., 2016). This avian species shows red astaxanthin-based coloration in the eye rings, bills and legs (García-de Blas et al., 2011, 2013, 2014, 2015). The diet of partridges was supplemented with the main astaxanthin precursor zeaxanthin and lutein (both yellow-orange carotenoids). An alternative treatment included astaxanthin only. Zeaxanthin-supplemented birds clearly increased coloration and astaxanthin level in ornaments, and the effect was even stronger when birds were exposed to a free-radical generator in water (diquat; see e.g. Galván and Alonso-Alvarez, 2009; Koch and Hill, 2017). Surprisingly, however, astaxanthin was not detected in the blood of astaxanthin-only treated birds, which also showed lower circulating levels of zeaxanthin and lutein, lower astaxanthin levels in ornaments and a paler color (García-de Blas et al., 2016).

In the present study, we aimed to go deeper into these potential interactions by testing the role of the other main ketocarotenoid in avian coloration: canthaxanthin. Canthaxanthin is less abundant in red-legged partridge ornaments than astaxanthin and shows lower (García-de Blas et al., 2013, 2014) or similar (this study) levels to papilioerythrinone (another ketocarotenoid less frequent in birds; e.g. Stradi et al., 2001). We also note that circulating canthaxanthin has not been detected in wild red-legged partridges (table 9S in García-de Blas et al., 2013), which suggests that it is not present in their natural diet or, if it is, it should be at very low amounts. In spite of its apparent scarcity and the fact that it would probably only be obtained naturally by ingesting aquatic organisms (see above), canthaxanthin is usually added in the commercial food of partridges and other bird species. In domestic chicken (*Gallus gallus*), canthaxanthin is commonly applied to intensify red colors and obtain more attractive products (e.g. Surai, 2012a,b). It should be noted that no sign of poor health or reproductive problems has been described as a consequence of this apparently unnatural diet (mostly the opposite; e.g. Weber et al., 2013; Karadas et al., 2016). Thus, although reduced liver vitamin A levels have been shown after canthaxanthin supplementation (table 7 in Karadas et al., 2016), canthaxanthin increases hatchability (Rosa et al., 2012), hen antioxidant status and skin coloration (Zhang et al., 2011), and also reduces oxidative damage in the embryo (Zhang et al., 2011; Rosa et al., 2012). The last would be explained by the antioxidant properties of carotenoids (e.g. McGraw et al., 2005; Pérez-Rodríguez, 2009; Surai, 2012a), though this function is still under debate at least in birds (e.g. Costantini and Møller, 2008; Koch et al., 2018).

In the present study, we aimed to test the impact of dietary canthaxanthin on red-legged partridge coloration and also tissue and egg yolk levels of other carotenoids and vitamins in order to infer potential interactions and also effects on reproductive output. We compared those control birds analyzed in García-de Blas et al. (2016) with a group of partridges that were fed with a common commercial canthaxanthin-supplemented diet in the course of (i.e. simultaneously with) the cited experiment. In addition, as described in García-de Blas et al. (2016), half of the birds included in the dietary carotenoid or control groups were exposed to a free radical generator in drinking water (i.e. diquat; see also Galván and Alonso-Alvarez, 2009; Koch and Hill, 2017) in order to test whether carotenoids act as an antioxidant, buffering oxidative stress and coloration loss (e.g. Hartley and Kennedy, 2004; Pérez-Rodríguez, 2009; Pérez-Rodríguez et al., 2010; Surai, 2012a,b). In our previous study (García-de Blas et al., 2016), we did not detect such an effect by testing zeaxanthin, lutein or astaxanthin supplements. Here, we also tested this hypothesis, but for canthaxanthin. We must note that canthaxanthin is considered a better antioxidant than astaxanthin, but weaker with regard to lutein or zeaxanthin (e.g. Mortensen and Skibsted, 1997).

MATERIALS AND METHODS

Experimental design and procedure

Here, we compared captive red-legged partridges, *Alectoris rufa* (Linnaeus 1758), used as the control group of another experiment (i.e. García-de Blas et al., 2016) with a group receiving a diet similar to commercial mixtures designed to intensify red coloration. Accordingly, the latter group of birds received the control diet but supplemented with canthaxanthin. The experiment described in García-de Blas et al. (2016) manipulated other carotenoids in the diet (i.e. lutein, zeaxanthin and astaxanthin) at higher concentrations than the canthaxanthin levels used here. This prevented a direct comparison of the two datasets but allowed us to infer the role of common canthaxanthin supplements on avian coloration.

This work was carried out at the Dehesa de Galiana experimental facilities (Instituto de Investigación en Recursos Cinegéticos and Diputación Provincial, Ciudad Real, Spain). It was conducted on captive-born, 1 year old red-legged partridges provided by a governmental breeding facility (Chinchilla, Albacete, Spain). Here, 62 adult partridges forming 32 pairs were kept in outdoor cages (1×0.5×0.4 m, each pair) under natural photoperiod and temperature. No bird died during the study, but two females were removed from the statistical analyses because they escaped during handling (i.e. one canthaxanthin+diquat and one control+diquat bird). In these cases, replacement birds were incorporated to keep pairs in similar conditions, but the new birds were not analyzed. The sex of individuals was determined genetically following Griffiths et al. (1998). Pairs were randomly divided into two groups that received one of the two diets. The final sample size for control birds was 45 birds from 23 pairs, and for canthaxanthin-supplemented birds it was 17 birds from 9 pairs. The imbalanced sample size was addressed statistically by testing the homoscedasticity assumption, which was always met. The experiment was carried out during the reproductive period (April–June); that is, when the color expression of the integuments is greatest (Pérez-Rodríguez, 2008). As they were yearlings, only some of the females laid eggs. Laying date and order, and the number of eggs laid per female were determined and analyzed (see 'Statistical Analysis', below, and Results). Moreover, one egg of each laying sequence was randomly chosen and analyzed for carotenoid and vitamin composition. The laying order of these eggs did not differ between the two carotenoid treatment groups (Student's t-test=0.915, d.f.: 11, P=0.380). All the eggs were removed daily from their cages and maintained in an incubator until hatching. The hatching rate (excluding the sampled egg) was also determined.

On 11 April ('day 0'), a blood sample and a color measurement (below) of each red ornament (eye ring, bill and legs) from each partridge were taken to determine pre-treatment color and blood levels of pigments and other physiological variables (below). A final color measure and blood sampling was performed at the end of the experiment (2 July; 'day 82'). For each blood sample, 1 ml of blood was taken from the jugular vein using heparinized syringes and centrifuged at 10,000 ***g*** for 10 min at 4°C to separate plasma from the cell fraction; both were stored separately at −80°C for later

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analysis. Before centrifugation, an aliquot of each blood sample was taken to calculate the hematocrit and resistance of erythrocytes against oxidative challenge (see below).

On 30 May, 15 pairs were randomly allocated to the oxidative challenge. Of these, 11 pairs were control birds and 4 pairs were canthaxanthin-treated birds. All these birds were treated with diquat dibromide, added to the drinking water. Diquat dibromide is a redox cycler that is transformed into a free radical which, in reaction with molecular oxygen, produces superoxide and other redox products (e.g. Sewalk et al., 2000; Zeman et al., 2005; Xu et al., 2007). The diquat bromide dose [0.50 ml l^{-1} Reglone (Syngenta, Madrid) in drinking water; Reglone contains 20% w/v diquat dibromide in water] was established on the basis of a pilot study and the results obtained in previous work in the same species, which reported no body mass changes but an increase in lipid oxidative damage in erythrocytes (see supporting fig. 1 in Alonso-Alvarez and Galván, 2011; see also Galván and Alonso-Alvarez, 2009).

At the last sampling event, all the birds were killed by cervical dislocation in order to take samples from internal tissues and ornaments. The experimental protocol was approved by the University of Castilla-La Mancha's Committee on Ethics and Animal Experimentation (CEEA, UCLM, Spain; reference number 1011.01).

Manipulation of carotenoid content in food

The manipulation of carotenoid content in the pellets was made on a basal commercial diet normally used during reproduction of captive red-legged partridges, containing wheat, barley, corn and soy in different proportions (INALSA, Ciudad Real, Spain). This feed did not contain any additional carotenoid to that naturally present in the grain (lutein and zeaxanthin; see below). To create the experimental canthaxanthin diet, a canthaxanthin supplement (Carophyll Red 10%, DSM Nutritional Products, Madrid, Spain) was added to the basal diet. This was made in collaboration with INALSA. All the pellets were produced following the usual method of commercial feed preparation by using large-scale mills (Pietsch, 2005). This process yielded perfectly homogeneous pellets, similar in size and color to base feed, avoiding pigmentation of the head of the birds by direct contact. To obtain the exact composition of carotenoids and vitamins in the pelleted food, HPLC-DAD (high-performance liquid chromatography with diode-array detection) analysis was carried out. The pigment in pellet samples was extracted by means of successive application of acetone and hexane. The hexane solution was then centrifuged (3000 ***g***, 5 min) and hexane extracts were evaporated and dissolved in an adequate amount of HPLC mobile phase for their identification and quantification (see description in 'Quantification of carotenoids and vitamins', below).

The carotenoid, tocopherol and retinol content of each type of pellet is shown in Table 1. The canthaxanthin diet included a higher total carotenoid level, which was mostly due to canthaxanthin. Unexpected higher levels of other carotenoids and retinol in the canthaxanthin diet were probably due to the protective antioxidant action of canthaxanthin during the pelleting process, which involves high pressure and temperature (Pietsch, 2005). Manipulation of carotenoid levels in the diet should resemble natural scenarios (Koch et al., 2016). However, the natural carotenoid content in the diet of wild red-legged partridges is currently unknown. We should also consider that body carotenoid levels of wild partridges are significantly higher than levels in captive birds that usually receive carotenoid supplements (table 9S in García-de Blas et al., 2015). This suggests that our supplements would not produce unnatural phenotypes. Moreover, no negative effect due to a hypothetical pharmacological level of carotenoids or diquat exposure was detected in terms of survival or body mass (see Results).

Quantification of carotenoids and vitamins

The quantification of carotenoids, retinol, retinyl and tocopherol in internal tissues (i.e. blood plasma and liver), egg yolks and colored integuments was performed by HPLC-DAD-fluorescence detection (FLD) following the methods described by Rodríguez-Estival et al. (2010) and García-de Blas et al. (2011, 2013). Carotenoid levels are total values including the levels of esterified and free forms for each specific pigment. Retinyl levels were only detected in the liver. We added retinyl values to retinol concentrations in order to obtain a measure of vitamin A in this organ. Standards of lutein, zeaxanthin, canthaxanthin, astaxanthin, astaxanthin monopalmitate and astaxanthin dipalmitate were purchased from CaroteNature (Lupsingen, Switzerland). Retinyl acetate (used as an internal standard) and standards of retinol and α-tocopherol were provided by Sigma-Aldrich.

Resistance to hemolysis under free radical exposure

The resistance of red blood cells to hemolysis under exposure to a free radical generator was assessed. Whole blood was exposed to a temperature-controlled free radical challenge by adding 2,2-azobis-(aminodinopropane) hydrochloride (AAPH) (Rojas Wahl et al., 1998). A detailed description of this technique can be found in García-de Blas et al. (2016). The lysis of red blood cells was assessed with a microplate reader device (PowerWave XS2, Bio-Tek Instruments Inc., Winooski, VT, USA), which measures the decrease in optical density at 540 nm wavelength every few minutes. Blood samples of a different bird species (zebra finch, *Taeniopygia guttata*) assessed twice were repeatable (repeatabilities *sensu* Lessells and Boag, 1987; here and subsequently: r=0.84, P<0.001, n=43). Units are reported as minutes to hemolyze 50% of erythrocytes based on absorbance decline.

Plasma antioxidants and metabolites

The total antioxidant status (TAS) of blood plasma was analyzed to estimate the availability of circulating antioxidants. As the idea that this measure assesses all antioxidants is questionable (it mostly measures hydrosoluble antioxidants), the term 'total' was avoided, and 'plasma antioxidants' (PLAOX) is used instead. The procedure is based on Miller et al. (1993), modified by Cohen et al. (2007) and Romero-Haro and Alonso-Alvarez (2014). Repeatability calculated on other samples of red-legged partridges assessed twice was high (r=0.94, P<0.001, n=20; Galván and Alonso-Alvarez, 2009). Albumin, uric acid and triglyceride levels in plasma were determined with commercial kits (Biosystems SA, Barcelona, Spain) with an automated spectrophotometer (A25-Autoanalyzer, Biosystems SA).

Table 1. Composition of a sample of each pelleted food used in the experiment

Diet	Canthaxanthin	Lutein	Zeaxanthin	Total carotenoids	Tocopherol	Retinol
Control	0	1.33	0.69	2.02	8.3	2.5
Canthaxanthin	9.9	2.26	1.18	13.34	8.7	6.9

Data are mg kg^{-1}.

Lipid peroxidation

To assess lipid oxidative damage, malondialdehyde (MDA) levels were measured in blood plasma, liver and heart. Plasma and liver lipid peroxidation may be influenced by diquat exposure but also by carotenoid levels as carotenoids are present in birds at high levels in these tissues. The analysis of heart muscles was a way to estimate a potential impact on animal performance/survival. The measurements were carried out following the method described by Romero-Haro and Alonso-Alvarez (2014). Livers and hearts were homogenized in a stock buffer (1:10 w/v phosphate buffer 0.01 mol l^{-1} adjusted to pH 7.4 with HCl 37%). Aliquots of 50 µl of the samples (plasma, homogenized liver and heart samples, and standards) were then capped and vortexed for 5 s and were analyzed by means of HPLC-FLD as described in Romero-Haro and Alonso-Alvarez (2014). Zebra finch plasma samples assessed twice provided very high within-session (r=0.97, n=20, P<0.001) and between-session (r=0.98, n=20, P<0.001) repeatability (Romero-Haro and Alonso-Alvarez, 2014).

Color measurements

The coloration of eye rings and bills of red-legged partridges was assessed using a portable spectrophotometer (Minolta CM-2600D, Tokyo, Japan). The redness of each trait was estimated from hue values that were in turn calculated by using the formula of Saks et al. (2003), which takes into account the brightness (B) of different colors, i.e. hue=arctan{[(B_y−B_b)/B_T]/[(B_r−B_g)/B_T]}, where y, r, b and g represent reflectance within the yellow (550–625 nm), red (625–700 nm), blue (400–475 nm) and green (475–550 nm) range, and B_T is total brightness. B_T obtained from our spectrophotometer (360–700 nm) was always added as a covariate to models testing the hue (see 'Statistical analysis', below), as the original formula includes B_T in both numerator and denominator, thus canceling out its effect. The repeatability of triplicate spectrophotometric measurements was significant for both traits (r>0.60, P<0.001), with mean values for each sample being used.

Leg color was assessed by means of digital photographs (Nikon D-3100, Minato, Tokyo, Japan; see also García-de Blas et al., 2013) because the probe of our spectrophotometer did not adapt well to the leg surface (see also Alonso-Alvarez and Galván, 2011). Birds were placed in the same position under standardized indoor light conditions (Kaiser Repro Lighting Unit; Repro Base with lights RB260 2×11 W 6000K; Kaiser Fototechnik, Buchen, Germany) with the camera (Nikon D-3100) always set to the same focus and conditions. A red color chip (Eastman Kodak Company, Rochester, NY, USA) was placed close to the legs in order to control for subtle changes in environmental light, adding the hue values of the chip as a covariate to models testing leg color (see 'Statistical analysis', below). Pictures were analyzed by a technician blind to the bird's identity. The color intensity of the central area of one of the tarsi was determined in adults by recording mean red, green and blue values (RGB system; e.g. Alonso-Alvarez et al., 2008) using Adobe Photoshop CS3. Hue was determined after conversion of RGB values using the Foley and Van Dam (1984) algorithm. Repeatability of picture measurements taken twice from a different sample of red-legged partridges was high (r>0.90, P<0.001, n=71; Alonso-Alvarez and Galván, 2011). As lower hue values obtained from spectrophotometer measures or pictures indicated higher redness levels, the sign of the hue variables was reversed (multiplied by −1) to simplify interpretations. The term 'redness' was thus used to describe the hue inverse.

Statistical analysis

All the analyses were performed using SAS v9.3 and SPSS software. The treatment effects on the number of females producing eggs were calculated from contingency tables (χ^2 tests). The variability in the number of eggs per female, hatching rates and pigment and vitamin levels of one egg per laying female were all tested by non-parametric Mann–Whitney U-tests because of the lack of normality and low sample size. All these analyses on reproductive output were separately performed for the period where birds received the control or carotenoid-supplemented diet but not diquat, during carotenoid treatment plus diquat exposure or on the full dataset (the complete study period).

To analyze the effect of the canthaxanthin-based diet and diquat exposure on the variability of the last sampling values (day 82), generalized mixed models (PROC MIXED in SAS software) were used. Here, carotenoid and diquat treatments and sex were tested as fixed factors, testing their interactions. To control for any subtle initial bias, we first obtained the residuals of mixed models testing color and blood levels at the first sampling event as the dependent variables and any relevant covariate or random effects as independent terms. These residuals were then added as covariates to the definitive models. The initial value was always included in the model (even when non-significant) for coherence among traits. Several other covariates were also added to the models. As previously mentioned, the redness (inverse of hue) of the eye ring and the bill was always controlled for total brightness. Again, for coherence, this was done even when non-significant (see backward stepwise procedure, below). In the case of the leg, the redness of the red chip was also tested. In all the mixed models, plasma retinol levels in the last sampling event, as well as retinol (or vitamin A) levels in every internal tissue and ornament, were also added to control for the difference in this parameter in the experimental diet (Table 1). These retinol covariates were removed when non-significant. We also explored a way to control for lutein and zeaxanthin values in a similar way, but this did not influence the results. Note that contrary to the diet data shown in Table 1, canthaxanthin-treated animals showed lower lutein and zeaxanthin levels in blood or liver compared with controls. In models testing plasma MDA levels, plasma triglyceride levels were added to control for potential influences of lipid variability in the blood (Romero-Haro and Alonso-Alvarez, 2014; Romero-Haro et al., 2015; Pérez-Rodríguez et al., 2015). In models testing PLAOX, uric acid and albumin levels were simultaneously tested to control for the influence of recent food intake (Cohen et al., 2007). To control for subtle differences in reproductive investment, the total number of eggs at the end of the study or the number of eggs during only the diquat experiment was also tested as an alternative covariate (in different models) but did not report any significant contribution and is not shown here. The lag time (min) to start hemolysis was added as a covariate in models testing resistance to hemolysis. Finally, the identity of the bird nested into the identity of the cage and the laboratory session for each technique were included as random factors (P-values ranging from 0.006 to 0.48; see also García-de Blas et al., 2016).

We always avoided overfitting (see Forstmeier and Schielzeth, 2011) by testing alternative models instead of full saturated models. Alternative models were also tested by removing terms at P>0.05 by following a backward stepwise procedure. The last best-fitted model was also compared with alternatives using the Akaike information criterion (AIC), providing similar conclusions. In some cases, dependent variables were transformed into mathematical functions to attain a normal distribution. This is described for each test in the Results. Differences are always provided as least square means±s.e.m. from models; that is, considering random factors and any term in the final model. Pair-wise comparisons were done by

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means of LSD *post hoc* tests. Satterthwaite degrees of freedom were used in mixed models to take into account the difference in sample sizes among variables and groups. The datasets are available from the Digital CSIC repository (http://dx.doi.org/10.20350/digitalCSIC/8567). Tables S1–S4 show descriptive statistics for the main models reported in Results.

RESULTS

Reproductive output

Thirteen females produced eggs [43%; 9 control diet, 4 canthaxanthin diet; 6 water (diquat control), 7 diquat]. Note that birds were yearlings and therefore they were not all expected to lay eggs. No test reported significant differences among treatments (all χ^2 reported $P>0.30$). Similarly, among laying females, the number of eggs produced during each period or throughout the study did not differ among treatments, and the same applies to hatching success (all Mann–Whitney *U*-tests reported $P>0.30$).

Diquat-exposure effects

Diquat decreased tocopherol concentration in every ornament in the whole of the sample [Fig. 1A–C; bill: $F_{1,29.3}=9.67$, $P=0.004$; eye ring (log-transformed): $F_{1,59}=8.14$, $P=0.006$; leg (log-transformed): $F_{1,60}=4.17$, $P=0.046$]. In the case of the bill, sex was retained in the final model ($F_{1,30.1}=7.33$, $P=0.011$), with males showing higher values (least square means±s.e.m.: 0.082±0.004 and 0.066±0.004 µmol g^{-1} for males and females, respectively). Tocopherol levels were also lower in the liver of diquat-treated birds (Fig. 1D; $F_{1,31.1}=18.96$, $P<0.001$).

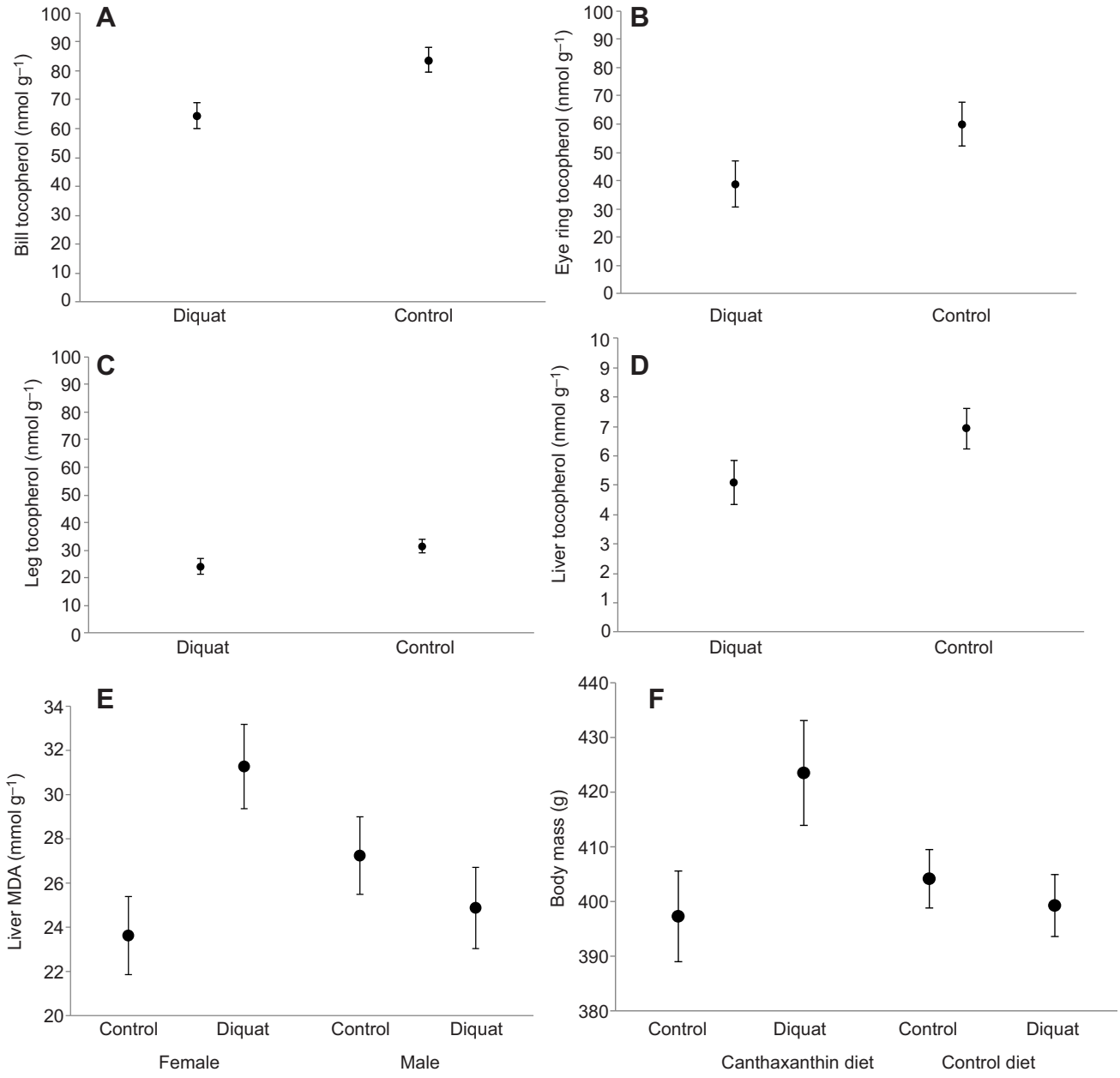

Fig. 1. Effects of diquat exposure on different parameters in red-legged partridges. (A–D) Tocopherol levels in ornaments (A, bill; B, eye ring; C, leg) and liver (D) following diquat exposure. (E) Liver oxidative damage (malondialdehyde, MDA) according to sex. (F) Body mass at the end of the study according to diet. Least square means±s.e.m. from the models (see Material and Methods, 'Statistical analysis').

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Liver lipid peroxidation (MDA) showed a significant interaction between diquat and sex (Fig. 1E; $F_{1,25.9}$=23.45, P<0.001). Females exposed to diquat showed higher oxidative damage in lipids compared with control females (P=0.006), whereas diquat-exposed males did not differ from controls (P=0.241). The model also included an interaction between the carotenoid group and sex ($F_{1,26.8}$=8.79, P=0.006). Here, among birds that did not receive canthaxanthin, females suffered higher oxidative damage than males (diet control females: 28.82±1.549 mmol MDA g^{-1}; diet control males: 24.03±1.572 mmol MDA g^{-1}; P<0.001). MDA levels of canthaxanthin-treated females and males did not differ (26.06±2.219 and 28.09±2.104 mmol g^{-1}, respectively; P=0.321). This could be due to canthaxanthin-treated males showing a trend towards increased MDA values compared with control males (P=0.073; Fig. 1E).

Finally, body mass variability showed an interaction between diquat and canthaxanthin effects (Fig. 1F; $F_{1,28.8}$=4.38, P=0.045). Canthaxanthin-treated birds exposed to diquat had higher body mass at the end of the study than dietary controls treated with diquat (P=0.038) and canthaxanthin-treated birds not exposed to diquat (P=0.047), and also showed a trend towards significance when compared with controls of both treatments (P=0.089). Other pairwise comparisons were clearly non-significant (all P>0.49). Diquat treatment did not show any significant influence in resistance to hemolysis, PLAOX or any other dependent variable (all P>0.10).

Dietary canthaxanthin increases canthaxanthin levels in partridges

Canthaxanthin levels were strongly increased in the blood plasma of canthaxanthin-treated animals (Fig. 2A; $F_{1,56.3}$=324.9, P<0.001; initial value: $F_{1,57}$=0.31, P=0.578). The same was found in the liver (Fig. 2B; $F_{1,42.4}$=39.08, P<0.001), vitamin A also being positively linked and included in the model ($F_{1,62.6}$=61.8, P=0.016; slope±s.e.m.: +3.43±1.38). Egg yolk also showed higher canthaxanthin concentrations (Fig. 2C; U=36.00, P=0.003). The canthaxanthin supplement also clearly increased canthaxanthin levels in the ornaments (Fig. 2D–F; bill: $F_{1,59}$=52.16, P<0.001; eye ring: $F_{1,54.4}$=73.26, P<0.001; leg: $F_{1,60}$=16.47, P<0.001).

Dietary canthaxanthin effects on other variables

Lutein values were lower in the plasma (Fig. 3A; $F_{1,56}$=42.19, P<0.001; initial value: $F_{1,56}$=4.73, P=0.034; plasma retinol: $F_{1,56}$=9.04, P=0.004; slope±s.e.m.: +0.127±0.042) and liver (Fig. 3C; $F_{1,58.6}$=38.47, P<0.001) of canthaxanthin-treated birds. Similarly, circulating levels of zeaxanthin were also lower in the canthaxanthin group than in the diet control group (Fig. 3B; $F_{1,56}$=43.23, P<0.001; initial value: $F_{1,56}$=3.13, P=0.083; plasma retinol: $F_{1,56}$=10.69, P=0.002; slope±s.e.m.: +0.090±0.028), the liver also showing lower concentrations (Fig. 3D; $F_{1,58.5}$=37.51, P<0.001). Lower lutein and zeaxanthin values were also found in the egg yolk (U=3.00, P=0.020 and U=4.00, P=0.031, respectively; Fig. 3E,F).

Canthaxanthin-treated birds also showed a trend to significantly higher retinol values in plasma compared with diet controls ($F_{1,30.3}$=3.52, P=0.070; 27.06±1.102 and 24.79 0.793 nmol ml^{-1}, respectively; initial value: $F_{1,47.9}$=4.27, P=0.044), and had double the liver vitamin A level of diet controls ($F_{1,60}$=26.44, P<0.001; 842.7±70.48 and 417.3±43.32 nmol g^{-1}, respectively). It should be noted that vitamin A levels were higher in the canthaxanthin diet (Table 1). In addition, the model testing diquat effects on liver tocopherol (above) also included an effect of carotenoid treatment ($F_{1,31}$=11.02, P=0.002). Canthaxanthin-treated partridges showed higher tocopherol values than controls (least square means±s.e.m.; 6.80±0.75 and 5.23±0.68 nmol g^{-1}, respectively). In contrast to vitamin A, we note that tocopherol values in the diets were only subtly higher in the canthaxanthin supplement. Finally, PLAOX showed a trend to significantly higher values in canthaxanthin-treated birds compared with diet controls [$F_{1,41}$=3.29, P=0.077; 0.754±0.033 and 0.683±0.020 (log-corrected values), respectively]. Nonetheless, a significant interaction between carotenoid treatment and sex arose when PLAOX values were controlled for levels of dietary metabolites (Fig. 4; $F_{1,35}$=5.99, P=0.013; uric acid: $F_{1,35}$=26.21, P<0.001; albumin: $F_{1,35}$=9.03, P=0.005; initial value: $F_{1,35}$=1.08, P=0.305). The result indicates that PLAOX increased in canthaxanthin-treated females only (this group differs from others at P<0.02). No other significant effect was detected in other parameters (all P>0.10).

Dietary canthaxanthin decreases red pigment levels and ornament redness

The canthaxanthin-treated red-legged partridges showed lower levels of the main red pigment (astaxanthin) of their non-feathered parts. The effect of diet was significant in the bill (Fig. 4A; $F_{1,58}$=25.23, P<0.001) and eye rings (Fig. 4B; $F_{1,54.4}$=4.63, P=0.036). The bill also revealed higher astaxanthin levels in males versus females ($F_{1,58}$=7.90, P=0.007; 2.97±0.26 and 1.96±0.28 µmol g^{-1}). In the model testing astaxanthin levels in the legs, carotenoid treatment only showed a trend towards lower astaxanthin values versus diet controls (Fig. 4C; $F_{1,28}$=2.94, P=0.097). The same model also showed a trend towards significance for lower astaxanthin levels in the legs of diquat-treated birds versus diquat controls ($F_{1,27.5}$=3.36, P=0.078; 0.089±0.058 and 0.220±0.052 µmol g^{-1}, respectively). Nonetheless, no term remained in the cited model after the backward stepwise procedure at P<0.05. Moreover, papilioerythrinone levels in any ornament did not reveal any significant effect of the canthaxanthin or diquat treatments or their interaction (all P>0.090).

Finally, and in agreement with astaxanthin variability, canthaxanthin-treated partridges showed lower redness in ornaments. The effect of the dietary supplement was significant in the bill (Fig. 4D; $F_{1,28.3}$=7.30, P=0.012; initial redness: $F_{1,56.6}$=2.48, P=0.121; total brightness: $F_{1,57.2}$=1.23, P=0.272) and eye rings (Fig. 4E; $F_{1,57}$=9.50, P=0.003; initial redness: $F_{1,57}$=7.81, P=0.007; total brightness: $F_{1,57}$=1.29, P=0.260; plasma retinol covariate: $F_{1,57}$=5.19, P=0.027; slope±s.e.m.: +1.771±0.778). In the case of the legs, the effect was a trend towards significance (Fig. 4F; $F_{1,28.7}$=3.41, P=0.075; initial redness: $F_{1,37.8}$=37.9, P<0.001; red chip: $F_{1,35.5}$=7.94, P=0.008). Here, the sex difference was also retained ($F_{1,29.6}$=6.35, P=0.017), with females showing redder legs than males (9.583±0.259 and 8.942±0.249, respectively).

DISCUSSION

Our results as a whole point to a strong interference of high levels of dietary canthaxanthin on the uptake of the main dietary yellow carotenoids (zeaxanthin and lutein). Zeaxanthin is considered an important precursor of astaxanthin, the main red carotenoid giving color to partridge non-feathered body parts (García-de Blas et al., 2016). Accordingly, birds treated with canthaxanthin showed lower astaxanthin levels in red ornaments and, hence, a paler coloration. No effect was found on papilioerythrinone in ornaments, i.e. the product of lutein transformation. We should, however, be cautious as we are here accepting the null hypothesis (lack of effect) despite using a low sample size (high risk of type II error). Moreover, papilioerythrinone is a minor carotenoid in red-legged partridge

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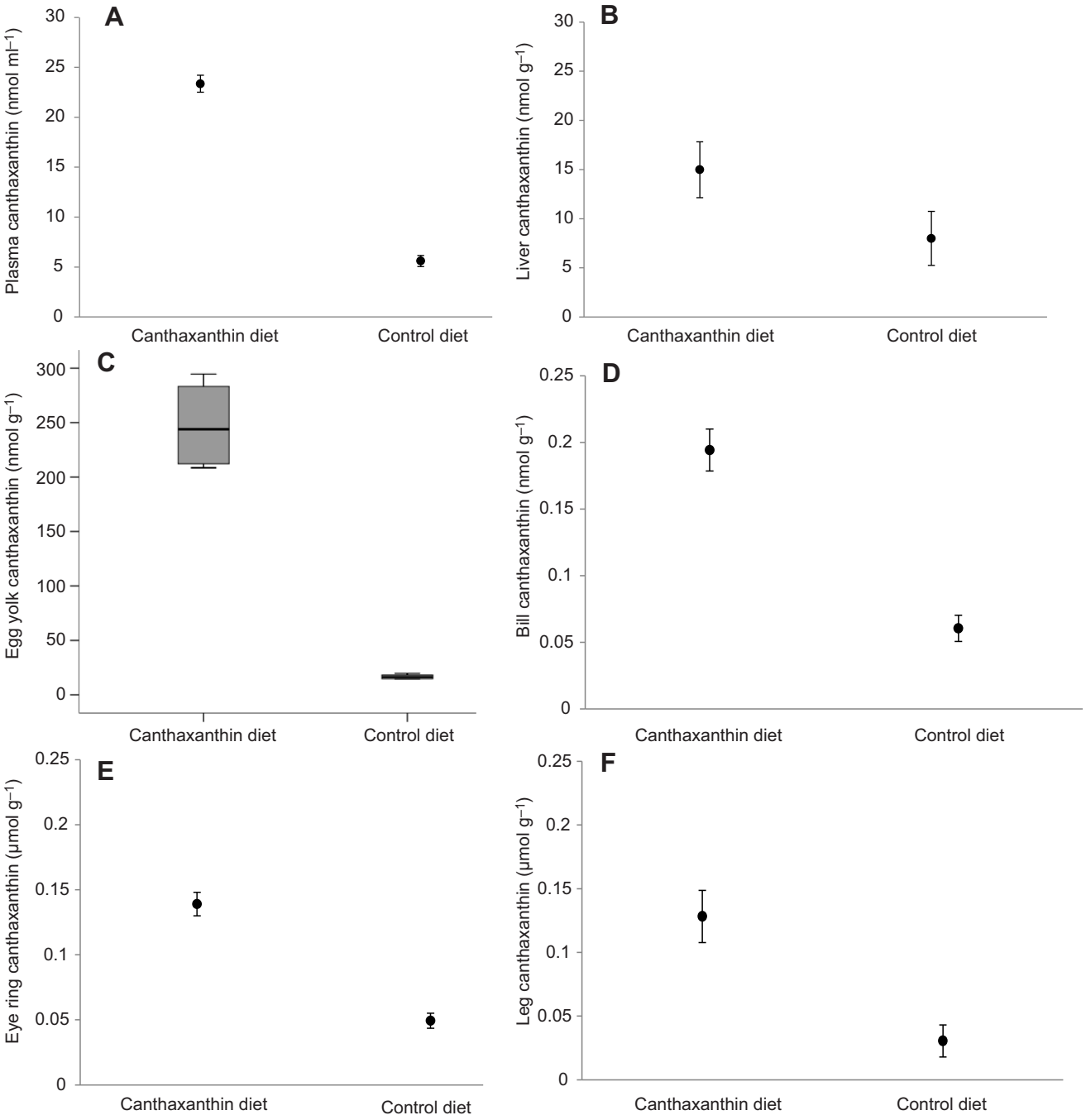

Fig. 2. Canthaxanthin accumulation in different tissues and eggs of red-legged partridges fed with a canthaxanthin-enriched diet compared with controls. Canthaxanthin levels in blood plasma (A), liver (B), egg yolk (C) and ornaments (D, bill; E, eye ring; F, leg). Least square means±s.e.m. from mixed models (A,B,D–F) and a box plot (C) showing medians, quartiles and range (see Materials and Methods, 'Statistical analysis').

coloration (García-de Blas et al., 2013, 2014). Nonetheless, our results are coherent with a strong competition for intestinal absorption between canthaxanthin and zeaxanthin, and warn of the indiscriminate use of the red pigment as a dietary supplement in birds. This scenario may also be present in the wild for birds fed on food items with an excess of those carotenoids not suitable for coloration. In that case, the results would contribute to understanding of the costs/benefits involved in red sexual signaling. Here, we must mention that captive male red-legged partridges whose red coloration was intensified by means of red paint obtained a better reproductive output as their mates laid more eggs (Alonso-Alvarez et al., 2012). Therefore, a decline in redness could impair partridge breeding success.

Canthaxanthin supplementation did not apparently buffer the oxidative stress associated with diquat, as *a priori* predicted. This, nonetheless, agrees with our previous results (García-de Blas et al., 2016), where no protective effect of any other supplemented carotenoid (i.e. lutein, zeaxanthin or astaxanthin) was found when birds were similarly treated with diquat. We should, however, note that astaxanthin was not absorbed into the blood in that study and, hence, its impact on tissue oxidative stress could not be tested. Regardless, these findings support the idea that the antioxidant effect of carotenoids

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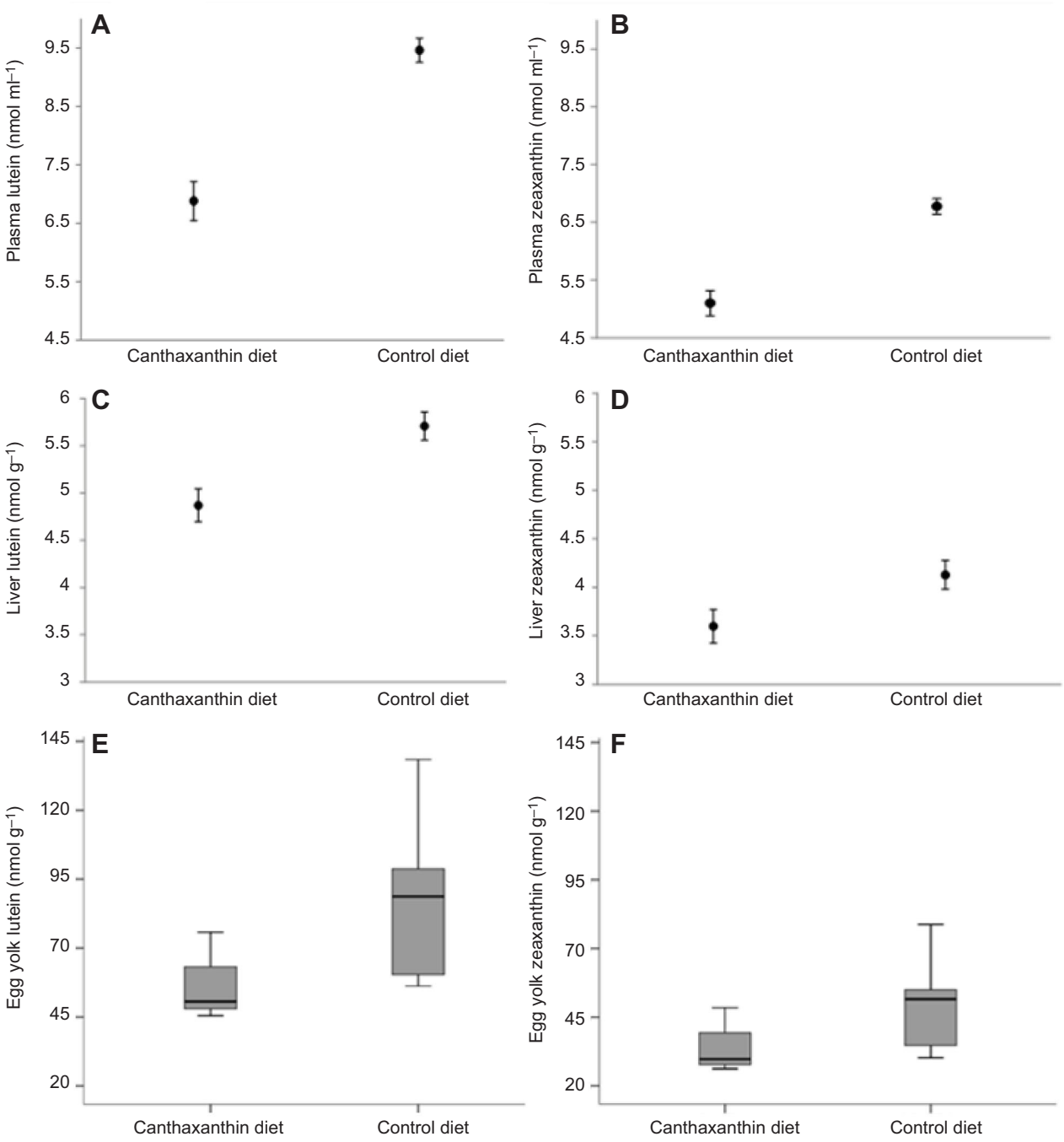

Fig. 3. Inhibition of lutein and zeaxanthin uptake by dietary canthaxanthin. Lutein (left) and zeaxanthin (right) levels in blood plasma (A,B), liver (C,D) and egg yolk (E,F). Least square means±s.e.m. from mixed models (A–D) and box plots showing medians, quartiles and ranges (E,F) (see Materials and Methods, 'Statistical analysis').

could be relatively minor at least for birds (e.g. Costantini and Møller, 2008; Pérez-Rodríguez, 2009; Koch et al., 2018). However, the results should be taken with caution as the reduced sample size could prevent detection of significant effects (avoiding type II error).

In agreement with its theoretical properties, diquat seems to have induced oxidative stress because vitamin E levels were lower in the three ornaments and liver of diquat-treated birds compared with controls. Diquat mostly acts by increasing superoxide anion levels (Koch and Hill, 2017) and tocopherol could have been used to quench this superoxide or lipid and hydrogen peroxides derived from superoxide actions (e.g. Sahin et al., 2002). Female partridges treated with diquat also exhibited higher lipid peroxidation in the liver compared with other groups (Fig. 1E). We could argue that female birds are more susceptible to oxidative stress as a result of the costs associated with antioxidant allocation to eggs (e.g. Williams, 2005; Romero-Haro et al., 2016). In fact, in another study, those female red-legged partridges laying eggs with higher hatchability (probably associated with antioxidant content; e.g. McGraw et al., 2005) showed higher lipid peroxidation in red blood cells (i.e. Alonso-Alvarez et al., 2010). Nonetheless, in our sample, liver MDA values were uncorrelated with egg number or hatchability in females ($P>0.20$). Finally, diquat-treated birds attained the largest body mass but only when fed with

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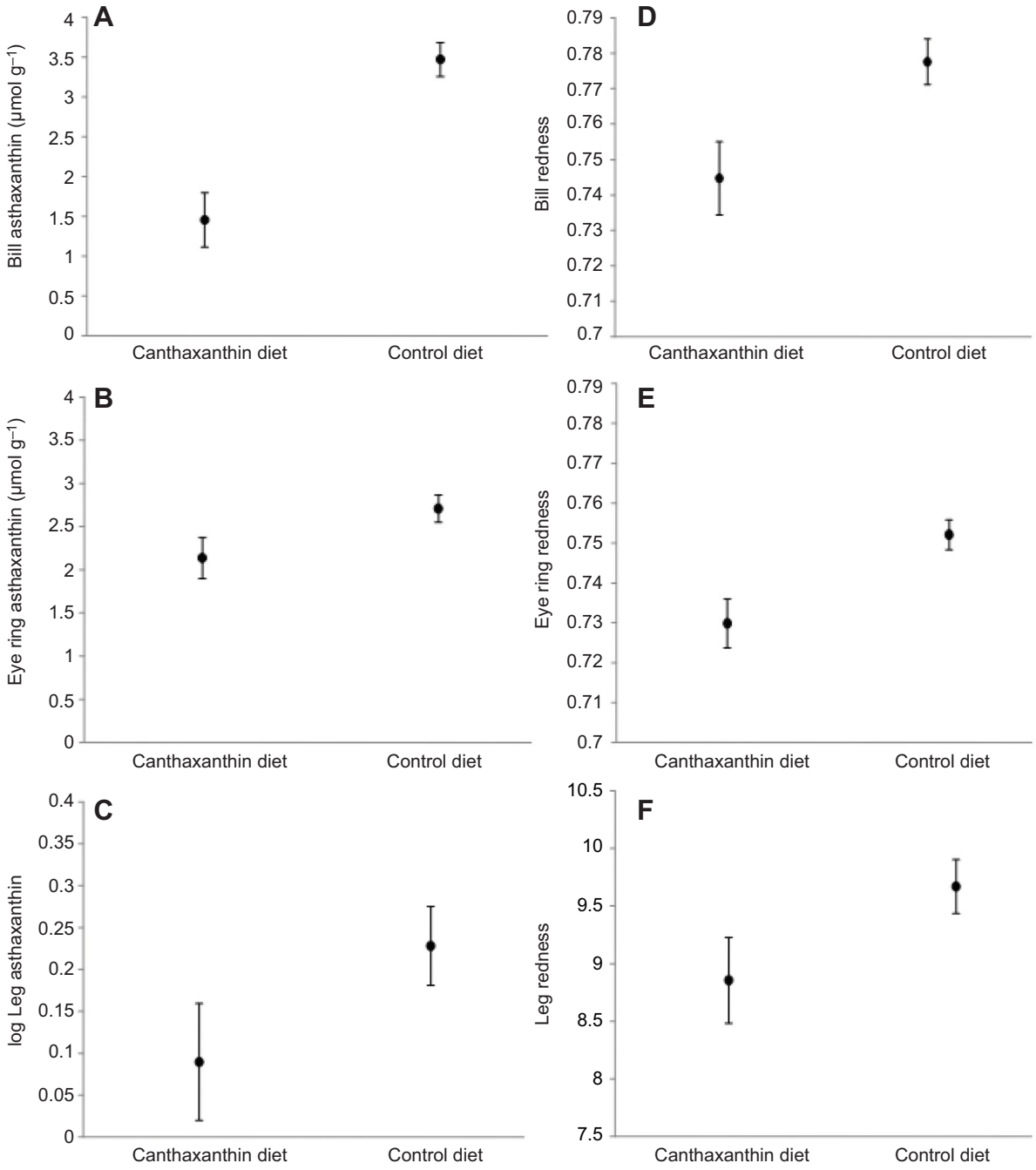

Fig. 4. Reduced red pigment levels in ornaments and paler traits in red-legged partridges fed with dietary canthaxanthin compared with controls. Astaxanthin levels (left) and redness (right) in bills (A,D), eye rings (B,E) and legs (C,F). Least square means±s.e.m. from the models (see Materials and Methods, 'Statistical analysis').

canthaxanthin (Fig. 1F). This is difficult to interpret, and may perhaps be linked to the antioxidant properties of canthaxanthin (e.g. Surai, 2012a,b; Esatbeyoglu and Rimbach, 2017; Johnson-Dahl et al., 2017), which could lead to a better overall condition and, ultimately, body mass. This, however, does not explain why canthaxanthin-treated birds did not show higher body mass in the absence of diquat.

However, the canthaxanthin supplementation experiment seems to show that this pigment, at least at high dietary concentrations, can exert a strong inhibition on yellow xanthophyll absorption, as previously shown for astaxanthin (García-de Blas et al., 2013). In contrast to astaxanthin, dietary canthaxanthin is apparently well absorbed because high levels were detected in blood, liver, egg yolk and also every red ornament (Fig. 2). Dietary canthaxanthin absorption has also been described in domestic chicken (Surai, 2012b) and some granivorous passerines (though inferred from tissue levels other that blood; see McGraw, 2006, and references therein). The competitive effect of dietary canthaxanthin can be deduced from the low values of zeaxanthin and lutein in the blood, liver and egg yolks of canthaxanthin-treated partridges (Fig. 3). The results contradict previous findings in green iguana, where very high levels of canthaxanthin in the diet (80 mg kg^{-1} food) did not impair yellow xanthophyll absorption but increased plasma levels

of lutein (Raila et al., 2002). In ferrets (*Mustela putorius furo*), a 2 year exposure to high dietary canthaxanthin (50 mg kg^{-1} body mass) led to a significant reduction of lutein and zeaxanthin levels in the fat, but not in other tissues (Tang et al., 1995). However, the same dose for 1 month did not produce significant effects on plasma lutein (Tang et al., 1993). In the case of birds, we have only been able to find one avian (poultry) study testing the effect of canthaxanthin supplementation on other circulating carotenoids, and this showed no effect on lutein values (Jensen et al., 1998). However, the cited study provided dietary canthaxanthin at similarly low levels to lutein (approximately 2.2 mg kg^{-1}; see also comment below). An interaction effect could, nonetheless, be deduced in another study in black-backed gulls (*Larus fuscus*) (Blount et al., 2002). The authors found that the high proportion of canthaxanthin in a carotenoid cocktail provided to females was also detected in the yolk of the eggs subsequently laid. However, the lutein, zeaxanthin and beta-carotene that were included in the same supplement were under-represented in the yolk. Unfortunately, although these results suggest a competitive effect of dietary canthaxanthin on carotenoid absorption, differential allocation and affinity for yolk lipids among carotenoids (Hencken, 1992) cannot be excluded.

In terms of proximate mechanisms, we can provide several possibilities. First, canthaxanthin could be better than yellow xanthophylls at crossing intestinal barriers. However, the higher polarity of lutein and zeaxanthin compared with canthaxanthin (Ramakrishnan and Francis, 1980; Xia et al., 2015) should have favored the yellow pigments during incorporation into mixed micelles and/or when crossing the enterocyte membranes (Furr and Clark, 1997; Maiani et al., 2009; Salter-Venzon et al., 2017, and references therein). Second, the different carotenoids could compete for transmembrane transport proteins present on the enterocyte surface (reviewed in Reboul, 2013). This theory is also unclear because, as far as we know, specific transport proteins have only been described for yellow xanthophylls or pro-vitamin A carotenoids, not for red ketocarotenoids (e.g. Hill and Johnson, 2012; Reboul, 2013). Thus, in some way, transmembrane transport proteins would be a route not shared by canthaxanthin and these compounds (i.e. no direct competition could exist), again favoring yellow pigments rather than canthaxanthin. Third, the lower absorption of lutein and zeaxanthin in the canthaxanthin-treated group could be the result of proportionally higher levels of canthaxanthin in the diet. Although lutein and zeaxanthin amounts in the experimental diet were double that of the control diet (Table 1), larger levels of canthaxanthin would have diluted yellow xanthophyll concentrations, reducing, in some way, their capacity to cross the intestinal barrier. Such an imbalance could also influence a last alternative: high carotenoid levels in the diet could stimulate their clearance, and this could be more evident in less represented (here yellow) pigments. High levels of canthaxanthin *in vitro* seem to induce increased activity of cytochrome P450 (i.e. CYPs) enzymes in the liver of rodents (Astorg et al., 1994, 1997; but see Zheng et al., 2013, for a reduction effect in humans).

Given the lack of knowledge of the proximate mechanism involved here, the possibility that red ketocarotenoids in the diet could reduce yellow xanthophyll levels in blood is relevant from an evolutionary perspective. It suggests that constraints during the simultaneous uptake of different pigments could have promoted the evolution of biotransformation (enzymatic) mechanisms to obtain red carotenoids from yellow ones, and finally produce red ornaments. The potential physiological costs derived from the interference in absorption (here as reduced lutein and zeaxanthin levels in tissues; see also García-de Blas et al., 2016) could also have promoted or reinforced that evolutionary process. However, the interference during uptake hypothesis requires the acceptance of two assumptions: (1) ketocarotenoids are available at high enough levels in the environment to be obtained from the diet and (2) animals cannot actively avoid those food items containing ketocarotenoids. The latter could be a consequence of main food resources always containing ketocarotenoids or individuals being unable to distinguish those food items containing high ketocarotenoid concentrations. In this regard, seed-based diets mostly contain lutein and zeaxanthin as principal carotenoids (e.g. McGraw, 2006, and references therein), red ketocarotenoids such as canthaxanthin and astaxanthin being mostly found in invertebrates (Kayser, 1982; Czeczuga, 1986). This opens the possibility of an active avoidance of those food items containing canthaxanthin when the physiological balance between costs and benefits becomes skewed to the first. The lack of detectable levels of canthaxanthin in the plasma of wild red-legged partridges (García-de Blas et al., 2013) may suggest that dietary ketocarotenoids are scarce or actively avoided in this species. In fact, partridges abundantly feed on cereals (e.g. Magalhaes et al., 2001; Holland et al., 2006) that do not contain ketocarotenoids (e.g. Panfili et al., 2004; Moore et al., 2005).

Alternatively, as commented in the Introduction, biotransformation mechanisms could have evolved as a consequence of ketocarotenoid scarcity in available food resources. In this scenario, the absence of absorption observed for astaxanthin (García-de Blas et al., 2016) or a competitive interaction between canthaxanthin and yellow pigments during uptake could be the consequence of the loss of physiological mechanisms responsible for an efficient transport from the food matrix to the blood. This loss would be due to relaxed or reversed selection on these mechanisms (e.g. Wiens, 2001; Porter and Crandall, 2003; Lahti, et al., 2009) as a consequence of the evolution of ketolases producing ketocarotenoids. This scenario may not particular to red-legged partridges. For instance, American flamingoes (*Phoenicopterus ruber*) fed with lutein or zeaxanthin are unable to absorb these two pigments, but are instead able to assimilate astaxanthin, which is used as a precursor for the main carotenoid in their feathers (i.e. canthaxanthin; Fox and McBeth, 1970; McGraw, 2006).

In summary, our results and those described in García-de Blas et al. (2016) showing an apparent inhibitory effect of dietary red ketocarotenoids on the absorption of yellow carotenoids has uncovered a possible reversed evolution of physiological pathways involved in the expression red carotenoid-based sexual signals. This selective process could be present in many other species. Future work should experimentally explore the capacity to absorb specific carotenoids in different model species, taking a comparative approach among different taxa. Our findings also highlight the importance of checking physiological constraints in new animal models before performing any dietary carotenoid supplementation. In this regard, attention should be paid to the proximate mechanisms involved. In particular, experiments manipulating the relative proportion of each carotenoid in the food are now necessary to discard or accept potential dilution mechanisms explaining uptake differences.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.A.-A.; Methodology: C.A.-A., E.G.-d.B., R.M.; Software: C.A.-A.; Validation: C.A.-A., R.M.; Formal analysis: C.A.-A.; Investigation: C.A.-A., E.G.-d.B., R.M.; Resources: C.A.-A., R.M.; Data curation: C.A.-A., E.G.-d.B.; Writing - original draft: C.A.-A.; Writing - review & editing: C.A.-A., R.M.; Visualization: C.A.-A.; Supervision: C.A.-A., R.M.; Project administration: C.A.-A., E.G.-d.B.; Funding acquisition: C.A.-A.

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Data availability

Data are available from the Digital CSIC repository (Alonso-Alvarez et al., 2018): http://dx.doi.org/10.20350/digitalCSIC/8567

Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.185074.supplemental

References

Alonso-Alvarez, C. and Galván, I. (2011). Free radical exposure creates paler carotenoid-based ornaments: a possible interaction in the expression of black and red traits. *PLoS ONE* **6**, e19403.

Alonso-Alvarez, C., Pérez-Rodríguez, L., Mateo, R., Chastel, O. and Viñuela, J. (2008). The oxidation handicap hypothesis and the carotenoid allocation trade-off. *J. Evol. Biol.* **21**, 1789-1797.

Alonso-Alvarez, C., Pérez-Rodríguez, L., García, J. T., Viñuela, J. and Mateo, R. (2010). Age and breeding effort as sources of individual variability in oxidative stress markers in a bird species. *Physiol. Biochem. Zool.* **83**, 110-118.

Alonso-Alvarez, C., Pérez-Rodríguez, L., Ferrero, M. E., García de-Blas, E., Casas, F. and Mougeot, F. (2012). Adjustment of female reproductive investment according to male carotenoid-based ornamentation in a gallinaceous bird. *Behav. Ecol. Sociobiol.* **66**, 731-742.

Alonso-Alvarez, C., García-de Blas, E. and Mateo, R. (2018). Data from: Dietary canthaxanthin reduces xanthophyll uptake and red coloration in adult red-legged partridges. *Digital CSIC Repository*. http://dx.doi.org/10.20350/digitalCSIC/8567.

Astorg, P., Gradelet, S., Leclerc, J., Canivenc, M.-C. and Siess, M.-H. (1994). Effects of beta-carotene and canthaxanthin on liver xenobiotic-metabolizing enzymes in the rat. *Food Chem. Toxicol.* **32**, 735-742.

Astorg, P., Gradelet, S., Leclerc, J. and Siess, M. H. (1997). Effects of provitamin A or non-provitamin A carotenoids on liver xenobiotic-metabolizing enzymes in mice. *Nutr. Cancer* **27**, 245-249.

Blount, J. D., Surai, P. F., Houston, D. C. and Møller, A. P. (2002). Patterns of yolk enrichment with dietary carotenoids in gulls: the roles of pigment acquisition and utilization. *Funct. Ecol.* **16**, 445-453.

Britton, G., Liaaen-Jensen, S. and Pfander, H. (2009). *Carotenoids. Volumen 5: Nutrition and Health*. Basel: Birkhäuser-Verlag.

Brown, E. D., Blakely, S. R., Babu, U., Grundel, E. and Mitchell, G. V. (1997). Vegetable concentrates interact with canthaxanthin to affect carotenoid bioavailability and superoxide dismutase activity but not immune response in rats. *Nutr. Res.* **17**, 989-998.

Choubert, G. (2010). Response of rainbow trout (Oncorhynchus mykiss) to varying dietary astaxanthin/canthaxanthin ratio: colour and carotenoid retention of the muscle. *Aquac. Nutr.* **16**, 528-535.

Cohen, A., Klasing, K. and Ricklefs, R. (2007). Measuring circulating antioxidants in wild birds. *Comp. Biochem. Physiol. B* **147**, 110-121.

Costantini, D. and Møller, A. P. (2008). Carotenoids are minor antioxidants for birds. *Funct. Ecol.* **22**, 367-370.

Czeczuga, B. (1986). The presence of carotenoids in various species of Lepidoptera. *Biochem. Syst. Ecol.* **14**, 345-351.

Esatbeyoglu, T. and Rimbach, G. (2017). Canthaxanthin: from molecule to function. *Mol. Nutr. Food Res.* **61**, 1600469.

Foley, J. D. and Van Dam, A. (1984). *Fundamentals of Interactive Computer Graphics*. Reading: Addison-Wesley.

Forstmeier, W. and Schielzeth, H. (2011). Cryptic multiple hypotheses testing in linear models: overestimated effect sizes and the winner's curse. *Behav. Ecol. Sociobiol.* **65**, 47-55.

Fox, D. L. and McBeth, J. W. (1970). Some dietary carotenoids and blood-carotenoid levels in flamingos. *Comp. Biochem. Physiol.* **34**, 707-713.

Furr, H. C. and Clark, R. M. (1997). Intestinal absorption and tissue distribution of carotenoids. *J. Nutr. Biochem.* **8**, 364-377.

Galván, I. and Alonso-Alvarez, C. (2009). The expression of melanin-based plumage is separately modulated by exogenous oxidative stress and a melanocortin. *Proc. R. Soc B* **276**, 3089-3097.

García-de Blas, E., Mateo, R., Viñuela, J. and Alonso-Álvarez, C. (2011). Identification of carotenoid pigments and their fatty acid esters in an avian integument combining HPLC-DAD and LC-MS analyses. *J. Chromat. B.* **879**, 341-348.

García-de Blas, E., Mateo, R., Viñuela, J., Pérez-Rodríguez, L. and Alonso-Alvarez, C. (2013). Free and esterified carotenoids in ornaments of an avian species: the relationship to color expression and sources of variability. *Physiol. Biochem. Zool.* **86**, 483-498.

García-de Blas, E., Mateo, R., Guzmán Bernardo, F. J., Rodríguez Martín-Doimeadios, R. C. and Alonso-Alvarez, C. (2014). Astaxanthin and papilioerythrinone in the skin of birds: a chromatic convergence of two metabolic routes with different precursors? *Naturwissenschaften* **101**, 407-416.

García-de Blas, E., Mateo, R. and Alonso-Alvarez, C. (2015). Accumulation of dietary carotenoids, retinoids and tocopherol in the internal tissues of a bird: a hypothesis for the cost of producing colored ornaments. *Oecologia* **177**, 259-271.

García-de Blas, E., Mateo, R. and Alonso-Alvarez, C. (2016). Specific carotenoid pigments in the diet and a bit of oxidative stress in the recipe for producing red carotenoid-based signals. *PeerJ* **4**, e2237.

Goodwin, T. (1984). *The Biochemistry of Carotenoids. Volumen II. Animals*. 2nd edn. Berlin: Springer-Verlag.

Griffiths, R., Double, M. C., Orr, K. and Dawson, R. J. G. (1998). A DNA test to sex most birds. *Mol. Ecol.* **7**, 1071-1075.

Hartley, R. C. and Kennedy, M. W. (2004). Are carotenoids a red herring in sexual display. *Trends Ecol. Evol.* **19**, 353-354.

Hencken, H. (1992). Chemical and physiological behavior of feed carotenoids and their effects on pigmentation. *Poultry Sci.* **71**, 711-717.

Hill, G. E. (2006). Color displays as intrasexual signals of aggression and dominance. In *Bird Coloration. Volume II. Function and Evolution* (ed. G. E. Hill and K. J. McGraw), pp. 137-200. Cambridge, Massachusetts: Harvard University Press.

Hill, G. E. and Johnson, J. D. (2012). The vitamin A-redox hypothesis: a biochemical basis for honest signaling via carotenoid pigmentation. *Am. Nat.* **180**, E127-E150.

Holland, J. M., Hutchison, M. A. S., Smith, B. and Aebischer, N. J. (2006). A review of invertebrates and seed-bearing plants as food for farmland birds in Europe. *Ann. Appl. Biol.* **148**, 49-71.

Hudon, J. and Brush, A. H. (1990). Carotenoids produce flush in the Elegant Tern plumage. *Condor* **92**, 798-801.

Isaksson, C. (2009). The chemical pathway of carotenoids: from plants to birds. *Ardea* **97**, 125-128.

Jensen, S. K., Jensen, C., Jakobsen, K., Engberg, R. M., Andersen, J. O., Lauridsen, C., Sørensen, S., Skibsted, L. H. and Bertelsen, G. (1998). Supplementation of broiler diets with retinol acetate, ß-carotene or canthaxanthin: effect on vitamin status and oxidative status of broilers in vivo and on meat stability. *Acta Agric. Scand. A* **48**, 28-37.

Johnson-Dahl, M. L., Zuidhof, M. J. and Korver, D. R. (2017). The effect of maternal canthaxanthin supplementation and hen age on breeder performance, early chick traits, and indices of innate immune function. *Poultry Sci.* **96**, 634-646.

Karadas, F., Erdoğan, S. I., Kor, D. I., Oto, G. I. and Uluman, M. (2016). Vitamin E and carotenoids in broiler diets on the growth performance, skin pigmentation and liver and plasma antioxidant concentrations. *Br. J. Poultry Sci.* **18**, 101-116.

Kayser, H. (1982). Carotenoids in stick insects (phasmids). *A quantitative comparison of six species at major developmental stages*. *Comp. Biochem. Physiol.* **72B**, 427-432.

Kiessling, A., Olsen, R.-E. and Buttle, L. (2003). Given the same dietary carotenoid inclusion, Atlantic salmon, *Salmo salar* (L.) display higher blood levels of canthaxanthin than astaxanthin. *Aquac. Nutr.* **9**, 253-261.

Koch, R. E. and Hill, G. E. (2017). An assessment of techniques to manipulate oxidative stress in animals. *Funct. Ecol.* **31**, 9-21.

Koch, R. E., Wilson, A. E. and Hill, G. E. (2016). The importance of carotenoid dose in supplementation studies with songbirds. *Physiol. Biochem. Zool.* **89**, 61-71.

Koch, R. E., Kavazis, A. N., Hasselquist, D., Hood, W. R., Zhang, Y., Toomey, M. B. and Hill, G. E. (2018). No evidence that carotenoid pigments boost either immune or antioxidant defenses in a songbird. *Nat. Commun.* **9**, 1-7.

Kostic, D., White, W. S. and Olson, J. A. (1995). Intestinal absorption, serum clearance, and interactions between lutein and β-carotene when administered to human adults in separate or combined oral doses. *Am. J. Clin. Nutr.* **62**, 604-610.

Lahti, D. C., Johnson, N. A., Ajie, B. C., Otto, S. P., Hendry, A. P., Blumstein, D. T., Coss, R. G., Donohue, K. and Foster, S. A. (2009). Relaxed selection in the wild. *Trends Ecol. Evol.* **24**, 487-496.

Lessells, C. M. and Boag, P. T. (1987). Unrepeatable repeatabilities: a common mistake. *Auk* **104**, 116-121.

Lopes, R. J., Johnson, J. D., Toomey, M. B., Ferreira, M. S., Araújo, P. M., Melo-Ferreira, J., Andersson, L. Hill, G. E., Corbo, J. C. and Carneiro, M. (2016).

Genetic basis for red coloration in birds reports genetic basis for red coloration in birds. *Curr. Biol.* **26**, 1427-1434.
Magalhaes, M. C., Tavres, P. and Fontoura, A. P. (2001). Morphometric characters and diet of hunted red-legged partridges (*Alectoris rufa*) in Portugal. *Game Wildlife Sci.* **18**, 3-4.
Maiani, G., Castón, M. J. P., Catasta, G., Toti, E., Cambrodón, I. G., Bysted, A., Granado-Lorencio, F., Olmedilla-Alonso, B., Knuthsen, P., Valoti, M. et al. (2009). Carotenoids: actual knowledge on food sources, intakes, stability and bioavailability and their protective role in humans. *Mol. Nutr. Food Res.* **53** Suppl. 2, S194-S218.
Matsuno, T. (2001). Aquatic animal carotenoids. *Fish Sci.* **67**, 771-783.
McGraw, K. J. (2006). Mechanisms of Carotenoid-based coloration. In *Bird Coloration. Volume I. Mechanisms and Measurements* (ed. G. E. Hill and K. J. McGraw), pp. 177-242. Cambridge, Massachusetts: Harvard University Press.
McGraw, K. J., Adkins-Regan, E. and Parker, R. S. (2005). Maternally derived carotenoid pigments affect offspring survival, sex ratio, and sexual attractiveness in a colorful songbird. *Naturwissenschaften* **92**, 375-380.
Micozzi, M. S., Brown, E. D., Edwards, B. K., Bieri, J. G., Taylor, P. R., Khachik, F., Beecher, G. R. and Smith, J. C., Jr. (1992). Plasma carotenoid response to chronic intake of selected foods and β-carotene supplements in men. *Am. J. Clin. Nutr.* **55**, 1120-1125.
Miller, N. J., Rice-Evans, C., Davies, M. J., Gopinathan, V. and Milner, A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Sci.* **84**, 407-412.
Moore, J., Hao, Z., Zhou, K., Luther, M., Costa, J. and Yu, L. L. (2005). Carotenoid, tocopherol, phenolic acid, and antioxidant properties of Maryland-grown soft wheat. *J. Agr. Food Chem.* **53**, 6649-6657.
Mortensen, A. and Skibsted, L. H. (1997). Relative stability of carotenoid radical cations and homologue tocopheroxyl radicals. A real time kinetic study of antioxidant hierarchy. *FEBS Lett.* **417**, 261-266.
Mundy, N. I., Stapley, J., Bennison, C., Tucker, R., Twyman, H., Kim, K.-W., Burke, T., Birkhead, T. R., Andersson, S. and Slate, J. (2016). Red carotenoid coloration in the zebra finch is controlled by a cytochrome P450 gene cluster. *Curr. Biol.* **26**, 1435-1440.
Negro, J. J., Tella, J. L., Blanco, G., Forero, M. G. and Garrido-Fernández, J. (2000). Diet explains interpopulation variation of plasma carotenoids and skin pigmentation in nestling White Storks. *Physiol. Biochem. Zool.* **73**, 97-101.
Paetau, I., Chen, H., Goh, N. M. and White, W. S. (1997). Interactions in the postprandial appearance of beta-carotene and canthaxanthin in plasma triacylglycerol-rich lipoproteins in humans. *Am. J. Clin. Nutr.* **66**, 1133-1143.
Panfili, G., Fratianni, A. and Irano, M. (2004). Improved normal-phase high-performance liquid chromatography procedure for the determination of carotenoids in cereals. *J. Agr. Food Chem.* **52**, 6373-6377.
Pérez-Rodríguez, L. (2008). Carotenoid-based ornamentation as a dynamic but consistent individual trait. *Behav. Ecol. Sociobiol.* **62**, 995-1005.
Pérez-Rodríguez, L. (2009). Carotenoids in evolutionary ecology: re-evaluating the antioxidant role. *BioEssays* **31**, 1116-1126.
Pérez-Rodríguez, L., Mougeot, F. and Alonso-Alvarez, C. (2010). Carotenoid-based coloration predicts resistance to oxidative damage during immune challenge. *J. Exp. Biol.* **213**, 1685-1690.
Pérez-Rodríguez, L., Romero-Haro, A. A., Sternalski, A., Muriel, J., Mougeot, F., Gil, D. and Alonso-Alvarez, C. (2015). Measuring oxidative stress: the confounding effect of lipid concentration in measures of lipid peroxidation. *Physiol. Biochem. Zool.* **88**, 345-351.
Pietsch, W. (2005). *Agglomeration in Industry*, Vol. 1. Weinheim: Wiley-VCH-Verlag.
Porter, M. L. and Crandall, K. A. (2003). Lost along the way: the significance of evolution in reverse. *Trends Ecol. Evol.* **18**, 541-547.
Raila, J., Schuhmacher, A., Gropp, J. and Schweigert, F. J. (2002). Selective absorption of carotenoids in the common green iguana (*Iguana iguana*). *Comp. Biochem. Physiol. A* **132**, 513-518.
Ramakrishnan, T. V. and Francis, F. J. (1980). Autoxidation of carotenoids and their relative polarity. *J. Food Qual.* **3**, 25-34.
Reboul, E. (2013). Absorption of vitamin A and carotenoids by the enterocyte: focus on transport proteins. *Nutrients* **5**, 3563-3581.
Rodríguez-Estival, J., Martínez-Haro, M., Martín-Hernando, M. P. and Mateo, R. (2010). Sub-chronic effects of nitrate in drinking water on red-legged partridge (*Alectoris rufa*): oxidative stress and T-cell mediated immune function. *Environ. Res.* **110**, 469-475.
Rojas Wahl, R. U., Zeng, L., Madison, S. A., DePinto, R. L. and Shay, B. J. (1998). Mechanistic studies on the decomposition of water soluble azo-radical-initiators. *J. Chem. Soc. Perkin Trans.* **2**, 2009-2018.
Romero-Haro, A. A. and Alonso-Alvarez, C. (2014). Covariation in oxidative stress markers in the blood of nestling and adult birds. *Physiol. Biochem. Zool.* **87**, 353-362.
Romero-Haro, A. A., Canelo, T. and Alonso-Alvarez, C. (2015). Early development conditions and the oxidative cost of social context in adulthood: an experimental study in birds. *Front. Ecol. Evol.* **3**, 35.
Romero-Haro, A. A., Sorci, G. and Alonso-Alvarez, C. (2016). The oxidative cost of reproduction depends on early development oxidative stress and sex in a bird species. *Proc. R. Soc. B* **283**, 80-86.
Rosa, A. P., Scher, A., Sorbara, J. O. B., Boemo, L. S., Forgiarini, J. and Londero, A. (2012). Effects of canthaxanthin on the productive and reproductive performance of broiler breeders. *Poultry Sci.* **91**, 660-666.
Sahin, K., Sahin, N., Yaralioglu, S. and Onderci, M. (2002). Protective role of supplemental vitamin E and selenium on lipid peroxidation, vitamin E, vitamin A, and some mineral concentrations of Japanese quails reared under heat stress. *Biol. Trace Elem. Res.* **85**, 59-70.
Saks, L., McGraw, K. and Hõrak, P. (2003). How feather colour reflects its carotenoid content. *Funct. Ecol.* **17**, 555-561.
Salter-Venzon, D., Kazlova, V., Izzy Ford, S., Intra, J., Klosner, A. E. and Gellenbeck, K. W. (2017). Evidence for decreased interaction and improved carotenoid bioavailability by sequential delivery of a supplement. *Food Sci. Nutr.* **5**, 424-433.
Senar, J. C. (2006). Color displays as intrasexual signals of aggression and dominance. In *Bird Coloration. Volume II. Function and Evolution* (ed. G. E. Hill and K. J. McGraw), pp. 87-136. Cambridge, Massachusetts: Harvard University Press.
Sewalk, C. J., Brewer, G. L. and Hoffman, D. J. (2000). Effects of diquat, an aquatic herbicide, on the development of mallard embryos. *J. Toxicol. Environ. Health A* **62**, 33-45.
Stradi, R., Pini, E. and Celentano, G. (2001). Carotenoids in bird plumage: the complement of red pigments in the plumage of wild and captive bullfinch (*Pyrrhula pyrrhula*). *Comp. Biochem. Physiol. B* **128**, 529-535.
Surai, P. F. (2012a). The antioxidant properties of canthaxanthin and its potential effects in the poultry eggs and on embryonic development of the chick. Part 1. *World's Poultry Sci. J.* **68**, 465-476.
Surai, P. F. (2012b). The antioxidant properties of canthaxanthin and its potential effects in the poultry eggs and on embryonic development of the chick. Part 2. *World's Poultry Sci. J.* **68**, 717-726.
Tang, G., Dolnikowski, G. G., Blanco, M. C., Fox, J. G. and Russell, R. M. (1993). Serum carotenoids and retinoids in ferrets fed canthaxanthin. *J. Nutr. Biochem.* **4**, 58-63.
Tang, G., Blanco, M. C., Fox, J. G. and Russell, R. M. (1995). Supplementing ferrets with canthaxanthin affects the tissue distributions of canthaxanthin, other carotenoids, vitamin A and vitamin E. *J. Nutr.* **125**, 1945-1951.
Twyman, H., Valenzuela, N., Literman, R., Andersson, S. and Mundy, N. I. (2016). Seeing red to being red: conserved genetic mechanism for red cone oil droplets and co-option for red coloration in birds and turtles. *Proc. R. Soc. B* **283**, 20161208.
Weber, G. M., Machander, V., Schierle, J., Aureli, R., Roos, F. and Pérez-Vendrell, A. M. (2013). Tolerance of poultry against an overdose of canthaxanthin as measured by performance, different blood variables and post-mortem evaluation. *Anim. Feed Sci. Tech.* **186**, 91-100.
White, W. S., Stacewicz-Sapuntzakis, M., Erdman, J. W. and Bowen, P. E. (1994). Pharmacokinetics of ß-carotene and canthaxanthin after ingestion of individual and combined doses by human subjects. *J. Am. Coll. Nutr.* **13**, 665-671.
Wiens, J. J. (2001). Widespread loss of sexually selected traits: how the peacock lost its spots. *Trends Ecol. Evol.* **16**, 517-523.
Williams, T. D. (2005). Mechanisms underlying the costs of egg production. *Bioscience* **55**, 39-48.
Xia, S., Tan, C., Zhang, Y., Abbas, S., Feng, B., Zhang, X. and Qin, F. (2015). Modulating effect of lipid bilayer-carotenoid interactions on the property of liposome encapsulation. *Colloids Surf. B.* **128**, 172-180.
Xu, J., Sun, S., Wei, W., Fu, J., Qi, W., Manchester, L. C., Tan, D.-X. and Reiter, R. J. (2007). Melatonin reduces mortality and oxidatively mediated hepatic and renal damage due to diquat treatment. *J. Pineal Res.* **42**, 166-171.
Zeman, M., Herichova, I., Navarova, J., Gressnerova, S. and Skrobanek, P. (2005). Melatonin interacts with effects of the herbicide diquat on selected physiological traits during ontogeny of Japanese quail. *Biologia* **60** Suppl. 17, 61-64.
Zhang, W., Zhang, K. Y., Ding, X. M., Bai, S. P., Hernandez, J. M., Yao, B. and Zhu, Q. (2011). Influence of canthaxanthin on broiler breeder reproduction, chick quality, and performance. *Poultry Sci.* **90**, 1516-1522.
Zheng, Y. F., Bae, S. H., Kwon, M. J., Park, J. B., Choi, H. D., Shin, W. G. and Bae, S. K. (2013). Inhibitory effects of astaxanthin, β-cryptoxanthin, canthaxanthin, lutein, and zeaxanthin on cytochrome P450 enzyme activities. *Food Chem. Toxicol.* **59**, 78-85.