Carotenoid biotransformation pathways and oxidative stress in a bird: an experimental approach and its implications for color signaling

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Colorful ornaments have been the focus of sexual selection studies since the work of Darwin. Yellow to red coloration is often produced by carotenoid pigments. Different hypotheses have been formulated to explain the evolution of these traits as signals of individual quality. Many of these hypotheses involve the existence of a signal production cost. The carotenoids necessary for signaling can only be obtained from food. In this line, carotenoid-based signals could reveal an individual's capacity to find sufficient dietary pigments. However, the ingested carotenoids are often yellow and became transformed by the organism to produce pigments of more intense color (red ketocarotenoids). Biotransformation often involves oxidation reactions. We tested the hypothesis that biotransformation could be costly because a certain level of oxidative stress is required. Thus, the carotenoid-based signals could reveal the efficiency of the owner in successfully managing this challenge. In a bird with ketocarotenoid-based ornaments (the red-legged partridge; Alectoris rufa), the availability of different carotenoids in the diet and oxidative stress were manipulated. We found that color and pigment levels in the ornaments depended on the relative quantity in the food of those carotenoids used as substrates in biotransformation (i.e. zeaxanthin and lutein). Moreover, we found that birds exposed to certain levels of a free radical generator (diquat) developed redder bills and deposited higher amounts of ketocarotenoids (astaxanthin) in ornaments, thus supporting the hypothesis. However, the effect also depended on the relative abundance of substrate carotenoids in the diet. This last result suggests the involvement of a resource allocation trade-off, which would support, to some extent, a signaling cost linked to carotenoid acquisition.

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Abstract

Colorful ornaments have been the focus of sexual selection studies since the work of Darwin. Yellow to red coloration is often produced by carotenoid pigments. Different hypotheses have been formulated to explain the evolution of these traits as signals of individual quality. Many of these hypotheses involve the existence of a signal production cost. The carotenoids necessary for signaling can only be obtained from food. In this line, carotenoid-based signals could reveal an individual's capacity to find sufficient dietary pigments. However, the ingested carotenoids are often yellow and became transformed by the organism to produce pigments of more intense color (red ketocarotenoids). Biotransformation often involves oxidation reactions. We tested the hypothesis that biotransformation could be costly because a certain level of oxidative stress is required. Thus, the carotenoid-based signals could reveal the efficiency of the owner in successfully managing this challenge. In a bird with ketocarotenoid-based ornaments (the red-legged partridge; *Alectoris rufa*), the availability of different carotenoids in the diet and oxidative stress were manipulated. We found that color and pigment levels in the ornaments depended on the relative quantity in the food of those carotenoids used as substrates in biotransformation (i.e. zeaxanthin and lutein). Moreover, we found that birds exposed to certain levels of a free radical generator (diquat) developed redder bills and deposited higher amounts of ketocarotenoids (astaxanthin) in ornaments, thus supporting the hypothesis. However, the effect also depended on the relative abundance of substrate carotenoids in the diet. This last result suggests the involvement of a resource allocation trade-off, which would support, to some extent, a signaling cost linked to carotenoid acquisition.

Keywords: Carotenoid supplementation, metabolic pathways, oxidative stress, avian coloration.

1. Introduction

Colored ornaments in animals have attracted the attention of evolutionary biologists since Charles Darwin, who suggested that most conspicuously colored traits are the product of sexual selection (Darwin 1871). Colored ornaments should provide some advantage when competing for a mate with same sex individuals (intrasexual selection) or by being more attractive to the choosing sex (intersexual selection; Andersson 1994). In many cases, colored traits inform competitors or potential mates about the quality of the owner. However, the trait should generate some benefit for both emitter and receptor to be considered as a signal (Hasson 1997; Bradbury & Vehrenkamp 1998; Maynar Smith & Harper 2003). This can occurs by the transmission of information in a reliable (non-falsifiable) way (Maynard Smith & Harper 2003).

In 1975, Amotz Zahavi proposed the "handicap principle", in which the reliability of the signal is due to its production/maintenance costs. The expression of a signal would proportionally be more costly for low-quality individuals compared to high-quality ones (Grafen 1990; also Getty 2006), the former being unable to signal or signaling in an inefficient way.

Carotenoids are natural pigments with immune-stimulant and antioxidant properties (Britton 2009) that are present in the integument of many vertebrate species, generating conspicuously colored traits (e.g. Brush 1990; Stradi 1998; McGraw 2006). The most obvious cost of carotenoid-based signals is the increase of conspicuousness that would raise the risk of predation (e.g. Godin & McDonough 2003). This idea was suggested as early as Darwin (1871), regarding colorful ornaments but without citing the pigments.

The second cost associated with these traits is related to the fact that carotenoids cannot be synthesized *de novo* by the organism, but are only obtained from food (Britton 2009; McGraw 2006). Assuming that carotenoids are relatively scarce in food, colored individuals should pay a cost in terms of energy or time spent searching for pigments, which was suggested by Endler (1980, 1983) in fish studies (also Kodric-Brown 1985; see in birds Hill 1990; McGraw 2006). This hypothesis is difficult to test and has garnered mixed support, at least in avian species (reviewed in Hill 2006), which is probably the

taxon where carotenoid-based signaling has been studied most in-depth (McGraw 2006; Pérez-Rodríguez 2009; Simons et al. 2012). Subsequently, Lozano (1994) was the first to emphasize the physiologically specific roles of carotenoids in an evolutionary context, suggesting that investing large amounts of pigment in signaling could compromise the immune system. This idea has been supported by results, particularly in birds (reviewed in Simons et al. 2012). Subsequently, von Schantz et al. (1999) followed a similar reasoning but regarding the antioxidant properties of the pigments, proposing that investing in coloration would challenge the individual's capacity to combat oxidative stress. This type of stress is the result of an imbalance between the production of reactive oxygen and nitrogen species (RONS) by cell respiration and immune responses and the state (levels and efficiency) of the antioxidant defenses (Halliwell & Gutteridge 2007). An evolutionary trade-off (Noordwijk & De Jong 1986) in the investment of the carotenoid resources between self-maintenance (antioxidant defense) and reproduction (sexual signaling) could thus be established (Møller et al. 2000; Alonso-Alvarez et al. 2008). The von Schantz et al. (1999) hypothesis has gained popularity (e.g. Blount et al. 2003; Alonso-Alvarez et al. 2004; Hörak et al. 2007), probably because it unifies the physiological components of trait expression, since the immune response is at least partially regulated by the oxidative machinery (Halliwell & Gutteridge 2007; Sorci & Faivre 2009; Vallverdú-Coll et al. 2015).

Nonetheless, the antioxidant role of those carotenoids involved in sexual signaling has been questioned. This criticism has mostly arisen from the weakness of some correlations between carotenoid blood levels and certain measures of antioxidant capacity or oxidative damage in avian species (Costantini & Møller 2008; Isaksson & Andersson 2008). However, a meta-analysis on the published literature of this taxon seems to support the carotenoid antioxidant function, although the results were not robust (Simons et al. 2012).

Importantly, the carotenoid molecules giving color to the ornaments are frequently not the same as those carotenoids obtained from the diet and circulating in the blood (e.g. fishes: Hata & Hata 1972; Ohkubo et al. 1999; birds: McGraw 2006 and references therein). This issue may be key to understanding the cost of the signal, but many obscure points are as yet not understood. In particular, the site (tissue) where carotenoids are

transformed and the type of biochemical processes involved in such transformations are little understood.

In avian species, the liver was the first tissue proposed as a potential biotransformation site (Brush & Power 1976; Brush 1990) because it stores large amounts of carotenoids and it is the main 'laboratory' of the organism (Blem 2000; Britton 2009). Carotenoid biotransformation in the liver could compete with the activity of enzymes involved in detoxification (Blem 2000; Hill and Johnson 2012). Hence, the fact that this vital organ could be involved could affect our understanding of the costs derived from color production. Carotenoid transformation in the liver was supported by studies in crossbills (*Loxia curvirostra*), which found the pigment used for coloration in the liver and blood (del Val et al. 2009a,b; see also Hill y Johnson 2012). Studies in many other bird species, however, did not find this and instead suggested that the ornament is the main transforming site (McGraw 2004, 2009; García de Blas et al. 2014), which would perhaps be less important for survival compared to the liver.

To understand how carotenoids are transformed we first need to know the biochemical route followed from substrate pigments to ornamental carotenoids, including the intermediate compounds (McGraw 2006; Britton 2009). Lutein and zeaxanthin are the most abundant carotenoids in the diet and blood of birds (McGraw 2006). Red ornaments displayed by many animal species are often the result of biotransformation of the cited yellow hydroxycarotenoids in red ketocarotenoids such as astaxanthin or canthaxanthin (McGraw 2006). The pathway followed from hydroxy- to ketocarotenoids requires hydrogenation and oxidation reactions. The existence of some specific enzymes (hydroxylases and 4-oxygenases [i.e. ketolases], respectively) has been proposed, but they have not been described in any vertebrate, at least for those species with carotenoid-based signals (McGraw 2006; Hill & Johnson 2012; Johnson & Hill 2013). Recently, Hill and Johnson (2012; Johnson & Hill 2013) have proposed that the oxidative status of the organism could influence the activity of these enzymes, with the carotenoid-based signals, in some way, revealing the individual's capacity to efficiently manage oxidative stress. The basic content of this idea was earlier formulated by Völker in 1957 when trying to explain why wild birds often lost their color in captivity. He proposed that this phenomenon is the

result of an impairment in the oxidative metabolism involved in carotenoid transformations. Although this could have deep implications for understanding the proximate costs of animal signaling, the hypothesis has not been experimentally tested until now.

In the present study, the red-legged partridge (*Alectoris rufa*) was used as the model species. This gallinacean shows red ornaments (bill, eye rings, and legs) mostly produced by astaxanthin and papilioerythrinone ketocarotenoids (García de Blas et al. 2013, 2014). We have experimentally shown that red head traits of males are used by females to adjust their reproductive investment, suggesting that these ornaments are indeed involved in sexual selection (Alonso-Alvarez et al. 2012). Experiments have also shown a relationship between integumentary coloration (and circulating carotenoid levels) and individual quality in terms of immune capacity (Pérez-Rodríguez and Viñuela 2008; Pérez-Rodríguez et al. 2008; Mougeot et al. 2009). Redder birds also show a better resistance to oxidative stress when exposed to an immune challenge (Pérez-Rodríguez et al. 2010). Moreover, young partridges exposed to high oxidative stress produced paler red traits and circulated lower blood carotenoid levels in adulthood (Alonso-Alvarez and Galván 2011). We have also described that astaxanthin and papilioerythrinone pigments are not present in blood, liver or fat, which indicates that pigment transformation takes place at the ornament site (García de Blas et al. 2013, 2014 and 2015). We have proposed that astaxanthin and papilioerythrinone should be derived from zeaxanthin and lutein in food, respectively (i.e. García de Blas et al. 2014), on the basis of published biochemical pathways (McGraw 2006; LaFountain et al. 2013). Lutein and zeaxanthin, in this order, are the most abundant carotenoids in the blood of this (García de Blas et al. 2013) and many other bird species (McGraw 2006). As previously noted, the biotransformation of these compounds should involve oxidative reactions (McGraw 2006). Dietary lutein would be transformed to papilioerythrinone after one 4-oxidation and one dehydrogenation reactions, whereas dietary zeaxanthin would be converted into astaxanthin by two 4-oxidations (McGraw et al. 2006; LaFountain et al. 2013, García-de Blas et al, 2014).

Here, the carotenoid content of the diet of captive red-legged partridges was manipulated, subsequently exposing birds to an oxidative challenge. Our aims were (1) to reveal the metabolic pathway from dietary carotenoids to those deposited in the ornaments,

(2) to verify the contribution to integument coloration of each dietary carotenoid, and (3) to determine if oxidative stress can influence color and the individual capacity to transform substrate carotenoids into those carotenoids allocated to ornaments. In this order, some birds received food supplemented with different zeaxanthin vs. lutein proportions, whereas other individuals received astaxanthin. In order to induce a higher oxidative stress, half of the birds in each treatment were also exposed to a free radical generator (diquat) in drinking water (Galvan & Alonso-Alvarez 2009). We first predicted that a higher proportion of zeaxanthin in the diet should increase astaxanthin levels in ornaments whereas a higher proportion of lutein should instead raise the papilioerythrinone concentration. Since astaxanthin is the most abundant pigment in ornaments (García de Blas et al. 2013, 2014), the group receiving dietary astaxanthin should *a priori* produce the reddest color and the highest astaxanthin concentrations in bare parts because no transformations would be required (Negro & Garrido-Fernández, 2000). If transformations depend on specific enzymes inducing oxidative reactions, the oxidative challenge (higher availability of free radicals) could perhaps favor these reactions, or instead, inhibit them by impairing/destabilizing the enzyme such as in the case of well-known antioxidant enzymes whose activity is decreased by high oxidative stress (e.g. glutathione synthase; Halliwell & Gutteridge 2007). In the first case, larger amounts of pigments in ornaments and redder colors should be expected, whereas the opposite would be true in the second scenario.

2. Material and Methods

2.1. Manipulation of carotenoid content in food

In order to manipulate the carotenoid content of the diet, we collaborated with a company dedicated to producing animal pelleted feed (INALSA; Ciudad Real, Spain; http://www.piensos-inalsa.com/contenido/perdices.htm). We preferred to manipulate carotenoid levels in food because carotenoids diluted in drinking water (1) can directly pigment head traits due to splashing (previous observations in this and other species) and (2) would have interfered with our oxidative stress manipulation. We supplied a free radical generator (diquat; see below) in water. Carotenoids and diquat in the same solution would have reacted producing pro-oxidant carotenoid metabolites (e.g. El-Agamey and

McGarvey, 2009). Alternatively, the use of two different water dispensers for each type of treatment would not have guaranteed a similar consumption of each solution.

The manipulation of carotenoid levels 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, Spain). This feed did not contain any additional carotenoid to those naturally present in the grain, and it was mixed with the different commercial carotenoids resulting in the final feed. Commercial pigments used to prepare the different diets for the experiment were CROMO ORO Classic (min. lutein 16 g/Kg and min. zeaxanthin 0.90 g/Kg), provided by DISPROQUIMA (Barcelona, Spain), OPTISHARP™ (Zeaxanthin 5% CWS/S-TG), provided by DSM Nutritional Products (Switzerland) and CAROPHYLL® Pink (Astaxanthin 10% CWS), provided by DSM Nutritional Products (Madrid, Spain). The adequate amounts of each pigment to add to the food were calculated taking into account the quantities of total carotenoids authorized for poultry feed (Directive 70/524/EEC, Communication 2004/C 50/01). Pellets were elaborated following the habitual 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 the pigmentation of the head of the birds by direct contact.

Diet 1 (Control) was the basal diet. Diets 2 and 3 contained lutein and zeaxanthin in different proportions: Diet 2 (called LutZea) contained approximately 73% lutein and 27% zeaxanthin, and diet 3 (ZeaLut) was formed by 52 % lutein and 48% zeaxanthin. Thus, diet 2 represented proportions often found in the natural diet of granivorous birds (McGraw 2006), whereas diet 3 was a diet enriched for zeaxanthin. Diet 4 was supplemented with astaxanthin (Ast). Carotenoid, tocopherol and retinol content of each type of pellet are shown in Table 1. Unexpected differences in tocopherol and retinol levels among treatments were found. This was probably due to the protective antioxidant action of carotenoids on vitamins present in the basal feed during the pelleting process, which involves high pressures and temperatures (Pietsch 2005), and to differences in the composition of supplements not detected during the formulation of each diet. To discard the influence of this potential bias, tocopherol and retinol levels in every analyzed tissue

(ornaments, plasma, liver and fat) were quantified and included as covariates in all statistical models (below).

2.2. Experimental procedure

The study was carried out at the Dehesa de Galiana experimental facilities (Instituto de Investigación en Recursos Cinegéticos and Diputación Provincial, Ciudad Real, Spain). The experimental protocol was approved by the University of Castilla-La Mancha's Committee on Ethics and Animal Experimentation. It was conducted on captive-born, one-year-old red-legged partridges provided by a governmental breeding facility (Chinchilla, Albacete, Spain). We used 182 adult partridges forming 91 pairs that were kept in outdoor cages (1 × 0.5 × 0.4 m, each pair) under natural photoperiods and temperatures. Ten birds were removed from the experiment (and statistical analyses) due to escapes during handling (treatment groups did not differ in these exclusions, all χ^2, $P > 0.12$). In these cases, replacement birds were incorporated to keep pairs in similar conditions, but the new birds were not included in posterior samplings. The sex of individuals was determined genetically following Griffiths et al. (1998). Pairs were randomly divided into four groups that received one of the four diets. The sample size for Control, LutZea and ZeaLut groups was 23 pairs, and 22 pairs for the Ast group. Possible differences between groups in terms of food intake were checked during the experiment by weighing the pellet mass in feeders of a subsample of 10 pairs per group during one week, with no difference being detected (repeated-measures ANOVA; $F_{3,80}$= 0.732, p = 0.536). The experiment was carried out during the reproductive period (April-June), when the color expression of integuments is the greatest (Pérez-Rodríguez 2008).

On April 11 ("time 1"), a blood sample and a color measurement (below) of each ornament (eye ring, bill, and legs) from each partridge was taken in order to determine pre-treatment color and blood levels of pigments and other physiological variables (below). Color measurements and blood samples were again taken on May 29 (day 48; intermediate sample or "time 2"). A third color and blood sampling was performed at the end of the experiment (July 2; day 82; "time 3"). One mL of blood was taken from the jugular vein, each time using heparinized syringes. Blood was centrifuged at 10,000 × g for 10 min at 4 ºC to separate plasma from the cell fraction. Both were stored separately at -80 ºC for later

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

On May 30, just after the second sampling, half of each treatment group (n = 45 pairs) were randomly allocated to the oxidative challenge. Of them, 11 pairs were from Control, ZeaLut, and Ast groups, and 12 pairs from the LutZea treatment. These birds were treated with diquat dibromide added to drinking water. The commercial product "Reglone" (Syngenta, Madrid) was used (20% w/v of diquat dibromide in water). Diquat dibromide is a redox cycler that is transformed to a free radical which, in reaction with molecular oxygen, produces superoxide and afterwards other redox products (e.g. Sewalk et al. 2001; Zeman et al. 2005; Xu et al. 2007). The diquat bromide dose (i.e. 0.50 mL/L Reglone in drinking water; Reglone contains 20% w/v of diquat dibromide in water) was established on the basis of a pilot study and the results obtained in previous work in the same species (see Supporting Fig.1 in Alonso-Alvarez and Galván, 2011).

2.3. Color measurements

Coloration of eye-rings and bills of red-legged partridges was assessed by using a portable spectrophotometer (Minolta CM-2600D, Tokyo). Hue values were calculated by using the formula of Saks et al. (2003) for brightness (B) of different colors (i.e. hue = arctan {[(By-Bb)/BT] / [(Br-Bg)/BT]}, where yellow [y] is the addition of percentage reflectance within the 550-625 nm range, blue [b] = 400-475 nm, green [g] = 475-550 nm and T is total brightness). BT obtained from our spectrophotometer (360-700 nm) was added as a covariate to models testing the hue (see Statistical Analyses), since the Saks et al. (2003) formula includes BT in both numerator and denominator, thus cancelling out its effect. Repeatabilities of triplicate spectrophotometric measurements were significant for both traits ($r > 0.68$, $P < 0.001$), with mean values for each sample being used.

Leg color was assessed by means of digital photographs (Nikon D-3100; see also García-de Blas et al. 2013) because the probe of our spectrophotometer did not adapt well to the leg surface (also Alonso-Alvarez & Galván 2011). In this case, the birds were placed in the same position under standardized indoor light conditions (Kaiser Repro Lighting

Unit; Repro Base with lights RB260 2x11W 6000ºK; Kaiser Fototechnik, Buchen) with the camera (Nikon D-3100) always set to the same focus and conditions. A red color chip (Kodak NY) 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 (*Statistical analyses*). Pictures were analyzed by a technician blind to the birds' 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 by 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 & Galvan 2011). Since lower hue values obtained from spectrophotometer measures or pictures indicated higher redness, 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.

2.4. Quantification of carotenoids and vitamins

The analyses of carotenoids, and vitamins A and E in internal tissues (i.e. plasma, liver, and subcutaneous fat) and colored integuments were performed by HPLC-DAD-FLD following the methods described by Rodríguez-Estival et al. (2010) and García-de Blas et al. (2011, 2013). Carotenoid levels are total values adding the levels of esterified and free forms for each specific pigment. 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. Carotenoid and vitamin concentrations were expressed as nmoles per gram of tissue.

2.5. 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 thermo-controlled free radical aggression by adding 2,2-azobis-(aminodinopropane) hydrochloride (AAPH) (Rojas Wahl et al. 1998). Previous work has shown that if at least one component of the antiradical detoxification system is impaired, the hemolysis curve shows a shift towards shorter times (Blache and

Prost 1992; Girard et al. 2005). This test, therefore, provides an assessment of resistance to oxidative stress because all families of free radical scavengers present in the blood are mobilized to fight off the oxidant attack (e.g. Blache and Prost 1992; Lesgards et al. 2002; Girard et al. 2005). Ten microliters of the blood of adult birds were immediately diluted and mixed in 365 µL of KRL buffer (for 50 mL: 0.020 g of $KHCO_3$; 0.0147 g of $CaCl_2$ $2H_2O$; 0.084 g of $NaHCO_3$; 0.4036 g of NaCl, 0.00746 g of KCl in 50 mL mili-Q water, adjusting pH to 7.4 with 3N HCl). The analyses were performed within 24 h following blood collection. Nonetheless, some aliquots could not be analyzed due to conservation problems, but this did not unbalance sample sizes of CAR and diquat treatments (all χ^2 tests: $P > 0.10$). Eighty microliters of KRL-diluted blood were incubated at 40 ºC with 136 µL of a 150 mM solution of AAPH. The lysis of red blood cells was assessed with a microplate reader device (PowerWave XS2, Bio-Tek Instruments Inc., Winooski, VT), which measures the decrease of optical density at the wavelength of 540 nm every few minutes. Blood samples of a different bird species (zebra finch, *Taeniopygia guttata*) assessed twice were repeatable ($r = 0.84$, $P < 0.001$, $n = 43$). Units are reported as minutes.

2.6. Plasma antioxidants

The total antioxidant status (TAS) of blood plasma was analyzed to estimate the availability of circulating hydrosoluble antioxidants. Since the idea that this measure assesses all the antioxidants is questionable, the term "total" was avoided, and hence, we will only use the generic "Plasma Antioxidants" (PLAOX). 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 & Alonso-Alvarez 2009).

2.7. Plasma biochemistry

Albumin, uric acid, triglycerides, LDL-cholesterol and total cholesterol levels in plasma were determined with commercial kits (Biosystems SA, Barcelona, Spain) with an automated spectrophotometer (A25-Autoanalyzer; Biosystems SA, Barcelona, Spain). The last two parameters were assessed to test for differences in lipid absorption due to diquat exposure (see Alonso-Alvarez & Galván 2011).

2.8. Lipid peroxidation

The measurement of lipid peroxidation in plasma, liver and heart was carried out following the method described in Romero-Haro and Alonso-Alvarez (2014). Livers and hearts were previously diluted (1:10 w/v) and were homogenized with a stock buffer (phosphate buffer 0.01 M 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 sec, and were analyzed 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 ($r = 0.98$, $n = 20$, $P < 0.001$) repeatabilities (Romero-Haro & Alonso-Alvarez 2014).

2.9. Statistical analyses

All the analyses were performed using SAS v9.3 software (SAS Institute 2006). The analyses are organized in two parts: (1) one testing the influence of carotenoid supplements only, and (2) the second analyzing the impact of the oxidative challenge (diquat exposure) and its interaction with carotenoid treatments.

The treatment effects on the number of birds producing eggs were calculated from contingency tables (χ^2). These analyses were separately performed for each experimental period (carotenoid exposure only or diquat exposure) and sex. Sex was considered because some females escaped during the experiment and hence sample sizes differed between sexes (see above). The variability in the number of eggs per individual was tested using a GENMOD procedure in the SAS software, including the number of eggs as a multinomial variable with cumulative logit link.

To test the carotenoid treatment (CAR hereafter) effect on color and blood variables throughout the study (i.e., three different measures), repeated-measures mixed models (PROC MIXED in SAS; Littell 2006) were used. In these models, the sampling event (TIME hereafter) was included as the repeated-measures factor, whereas the identity of the individual nested into cage identity was the subject term (REPEATED statement; Littell 2006). CAR (four-level factor), TIME (three-level factor) and sex were always included in the models as fixed effects, testing their two- and three-way interactions. Since the aim was

exclusively testing the CAR effect with the highest available statistical power, these repeated-measure models did not include data from those individuals exposed to diquat (time 3 only).

To analyze the effect of diquat, variability at time 3 was analyzed by generalized mixed models (PROC MIXED in SAS). Here, CAR and diquat treatments and sex were tested as fixed factors, testing their interactions. Color and blood levels at the precedent sampling event (time 2) were tested as covariates to correct for potential differences between groups at the start of the diquat exposure.

Other different covariates were added to the models. Thus, as previously mentioned, the redness (inverse of hue) of the eye ring and bill was controlled for total brightness. In the case of the leg, the redness of the red chip was tested. In all the repeated-measures mixed models testing the CAR effect, the influence of plasma vitamin (tocopherol and retinol) levels was tested by including them as covariates. In all the mixed models testing the diquat effect, plasma vitamin levels in the last sampling event, as well as vitamin levels in every internal tissue and ornaments, were also added. In models testing plasma MDA values, plasma triglyceride levels were added to control for potential influences of lipid variability in the blood (Romero-Haro & Alonso-Alvarez 2014; Romero-Haro et al. 2015). In models testing PLAOX, uric acid, and albumin values were simultaneously tested to control for influences of recent food intake (Cohen et al. 2007). To control for subtle differences in reproductive investment, the number of eggs produced at the end of each sampling interval ("eggs") was also tested as a covariate in repeated models (Table 2). In models testing final variability (Tables 3 and 4), the total number of eggs at the end of the study or the number of eggs during only the diquat experiment were tested as alternative covariates (in different models). The lag time (min) to start hemolysis and hematocrit were added as covariates in models testing resistance to hemolysis. Finally, the identity of the bird nested into the identity of the cage and the laboratory session were included as random factors (*P*-values ranging from <0.001 to 0.476).

All the mixed models were explored from the saturated models. They firstly included all the covariates (although see alternative options above), fixed factors, and factor interactions. Alternative models were then tested by removing terms at $P > 0.10$ by

following a backward-stepwise procedure. The last best fitted model was also compared to alternatives using the Akaike Information Criteria (AIC), providing similar conclusions. When tested as dependent variables, carotenoids and vitamins were transformed with mathematical functions to attain a normal distribution. All carotenoids and tocopherol levels were log-transformed, whereas vitamin A levels in the liver were transformed by a square root. In subcutaneous fat, carotenoid and retinol levels were standardized into two blocks because some sample sessions gave particularly low values. Differences are always provided as least squared means ± SE from models; that is, considering random factors and any term in the final model. Pair-wise comparisons were done by means of LSD *post hocs*. The description of interactions and their figures in the main text are restricted to tests reporting $P < 0.10$. Other models, figures and tables containing means and SD from raw data are described in the Supporting Material.

3. Results

3.1. Egg laying

The treatments did not affect the number of individuals producing eggs during the first (carotenoid supply only; all χ^2 tests: $P > 0.34$) or second (diquat × carotenoid supply interaction; all χ^2 tests: $P > 0.86$) part of the experiment. Similarly, the treatments did not influence the number of eggs produced during the first period (all χ^2 tests: $P > 0.65$) or the total number of eggs laid during the whole study (all χ^2 tests: $P > 0.11$). The addition of tocopherol or retinol values as covariates did not change any of these results. The tests on egg production reported similar results when including those males that were housed with new partners during the study (all tests: $P > 0.10$).

3.2. Influence of carotenoid treatments

Body mass was not affected by CAR treatments (time and sex interactions all $P > 0.90$). In contrast, integument coloration changed throughout the study according to carotenoid supplements. Redness decreased throughout reproduction, but the LutZea and ZeaLut groups counteracted this effect (CAR × time interaction) in the eye ring and bill, although the latter trait only showed a trend toward significance (Table 2; Fig.1). In the eye ring, ZeaLut birds were redder than control and Ast partridges at the second sampling (both $P <$

0.05; Fig. 1). On the last day, LutZea and ZeaLut groups showed redder eye rings than the other treatments ($P < 0.034$), but did not differ between them ($P = 0.411$; Fig. 1). In the bill, differences arose at the last sampling, with LutZea, ZeaLut (both $P < 0.001$) and control (but $P = 0.068$) birds redder than Ast animals. ZeaLut and LutZea birds were also redder than controls, with the latter only a trend ($P = 0.017$ and 0.064, respectively; LutZea vs. ZeaLut: $P = 0.673$; Table 2, Fig. 1SM). The legs did not show a significant interaction (Table 2), although ZeaLut birds were redder than controls at the second and last samplings (both $P < 0.013$; Fig. 1SM).

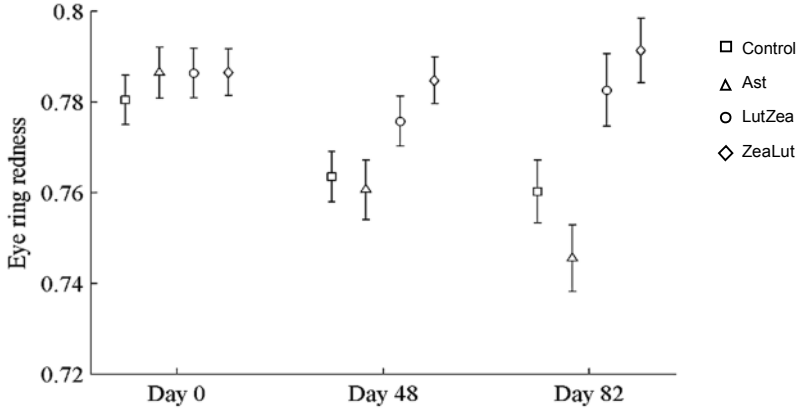

Figure 1. *Changes in eye ring coloration during the experiment depending on the carotenoid treatment. Least squared means ± se were obtained from the models (see Methods).*

In terms of plasma pigments, the carotenoid treatment interacted with time (Table 2 and Fig. 2). Lutein levels did not differ between ZeaLut and control birds at time 2 ($P = 0.48$), but the other comparisons among groups on that day and at the last sampling were highly significant (all $P < 0.001$), with LutZea birds showing the highest values (Fig. 2). In the case of zeaxanthin, only Ast and control birds did not differ at the last sampling ($P = 0.730$), with the other groups differing clearly (all P-values < 0.001). Agreeing with predictions, ZeaLut partridges showed the highest zeaxanthin values (Fig. 2).

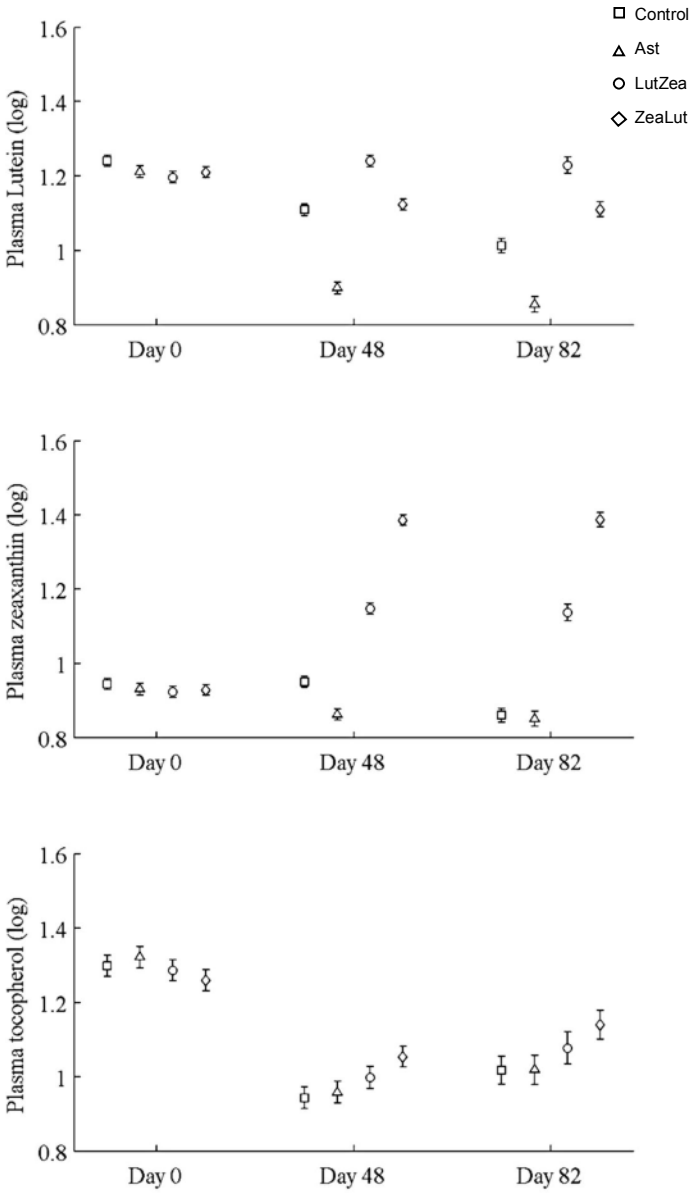

Figure 2. *Changes in plasma carotenoids and tocopherol levels (log-transformed) during the experiment depending on the carotenoid treatment. Least squared means ± se from the models (see Methods and Table 2).*

Plasma vitamins used as covariates in these models (Table 2) were also tested as dependent variables. The CAR × time interaction was not significant for retinol, but was for tocopherol (Table 2 and Fig. 2). ZeaLut birds showed higher tocopherol values than control and Ast individuals from 48 days to the end of the study (both $P < 0.020$; other comparisons: $P > 0.13$).

PLAOX changed according to the supplemented carotenoid (Table 2 and Fig. 3). On day 48, Ast showed higher values than other groups (all $P < 0.012$), with controls reporting higher mean levels than ZeaLut ($P = 0.034$) and LutZea (but $P = 0.098$) birds. At the last sampling, LutZea birds increased their values approaching Ast individuals ($P = 0.715$). Ast birds again differed from the other two groups (both $P < 0.023$), with LutZea animals showing a trend toward higher values than control ($P = 0.052$) and ZeaLut ($P = 0.080$) birds. The interaction remained ($P = 0.020$) when removing albumin and uric acid covariates (Fig. 3).

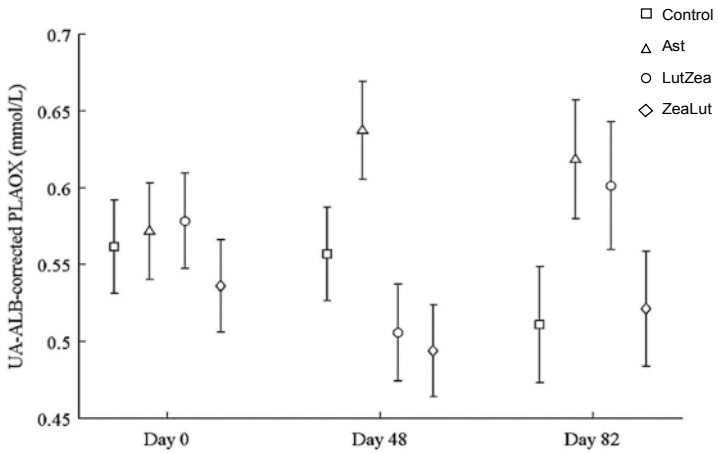

***Figure 3**: Changes in the levels (mmol/L) of plasma antioxidant status (controlled for albumin and uric acid levels) during the experiment depending on the carotenoid treatment. Least squared means ± se from the models (see Methods and Table 2).*

Finally, plasma MDA (i.e., corrected or uncorrected for plasma lipid levels) and the resistance to oxidative stress in erythrocytes did not show significant differences with CAR during the study (all P-values > 0.64; Table 2).

3.3. Variability after diquat exposure

Body mass controlled for tarsus length variability was not influenced by CAR or diquat treatments or their interactions (all $P > 0.10$). The same was found for circulating LDL- and total-cholesterol levels (all $P > 0.12$).

3.3.1. Ornament color and pigments

In terms of redness, CAR did not clearly interact with diquat in any trait (all $P > 0.24$; Table 3 and Fig. 2SM). Nonetheless, diquat-exposed birds showed marginally significant redder bills among control and ZeaLut birds ($P = 0.051$ and 0.084, respectively; Fig. 2SM). Moreover, in the eye ring model, sex showed a trend toward a significant interaction with diquat ($P = 0.069$ in its last backward step). Males showed redder eye rings than females, but only among diquat-treated pairs (post hoc: $P = 0.020$; diquat male: 0.770 ± 0.006; diquat female: 0.757 ± 0.006; control male: 0.762 ± 0.006; control female: 0.764 ± 0.006; other pairwise comparisons: $P > 0.18$). In any event, in the best-fitted model excluding any interaction (i.e. Table 4), the diquat treatment alone reported a significant effect on bill redness, with diquat-treated birds showing redder bills (Fig. 4).

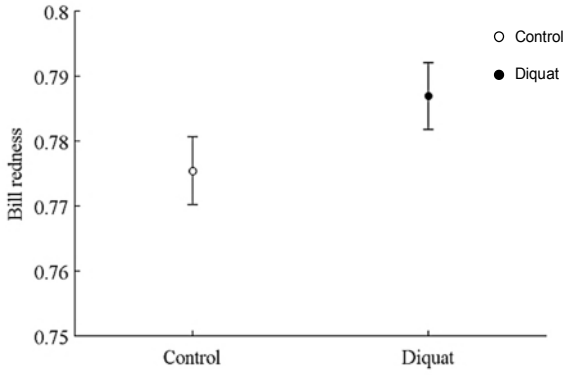

Figure 4. *Effect of the diquat treatment on bill redness. Least squared means ± se from the models (see Methods and Table 4).*

Best-fitted models for any ornament also showed a strong CAR effect (all *P-values* < 0.001; Table 4). Ast birds were always the palest individuals (all $P < 0.001$), whereas ZeaLut partridges were the reddest ones, followed by LutZea birds and controls (Fig. 5). Importantly, the difference in color between ZeaLut and LutZea animals was significant in eye rings and legs (both $P < 0.044$; in the bill: $P = 0.065$; Fig. 5).

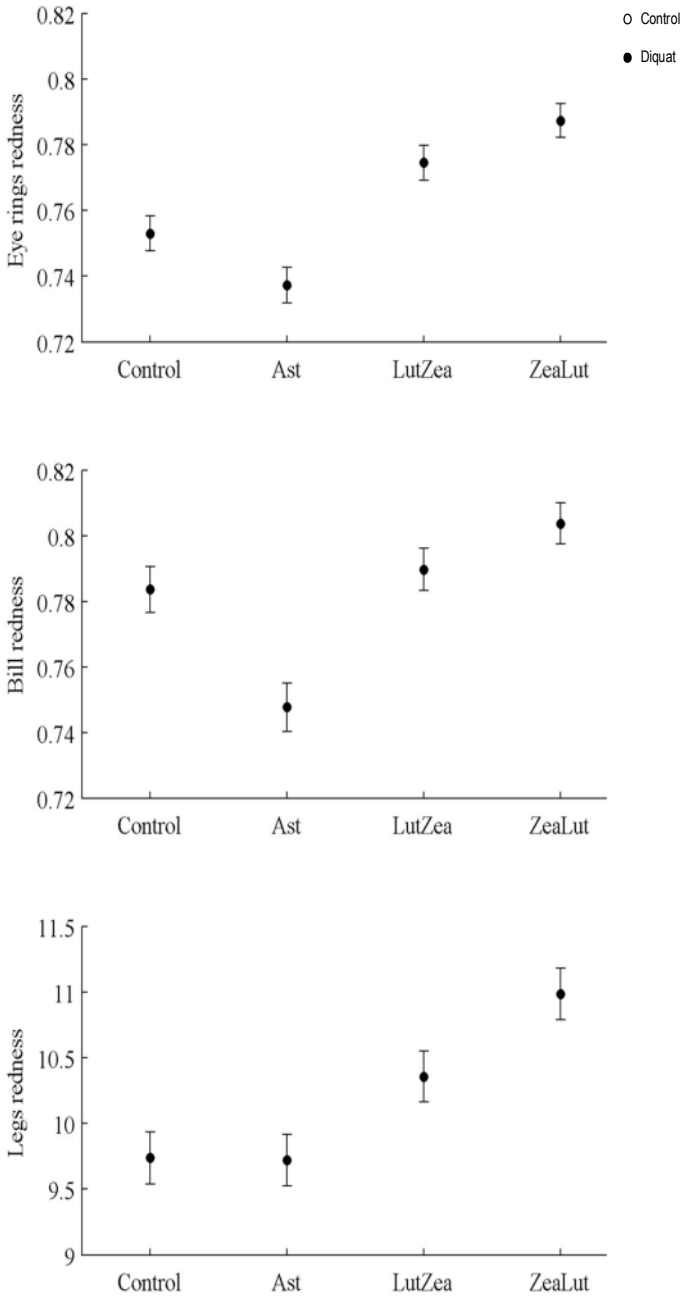

Figure 5. *Ornament coloration controlled for diquat treatment and integumentary redness at day 48. Least squared means ± se from the models (see Methods and Table 4).*

Concerning pigments, diquat affected astaxanthin levels in the eye ring and bill, but depending on CAR (Table 3 and Fig. 6). The effect was partially due to differences in CAR-controls of both traits (both $P < 0.020$), with diquat-treated birds showing higher astaxanthin concentrations. Nonetheless, in the eye rings ZeaLut birds showed a marginally significant difference in the same direction ($P = 0.057$). In the same eye ring and bill models, all pair-wise comparisons between carotenoid groups (CAR factor: both $P < 0.001$) were significant (all $P < 0.013$), showing increasing astaxanthin values in the following order: Ast, control, LutZea and ZeaLut (Fig. 6). In legs, the diquat × CAR interaction did not affect astaxanthin (Table 3). Only CAR remained in the model (Table 4), with LutZea and ZeaLut birds showing higher astaxanthin levels (Fig. 3SM) than other groups (all $P < 0.025$), but not differing between them ($P = 0.162$; also Ast vs. control: $P = 0.248$).

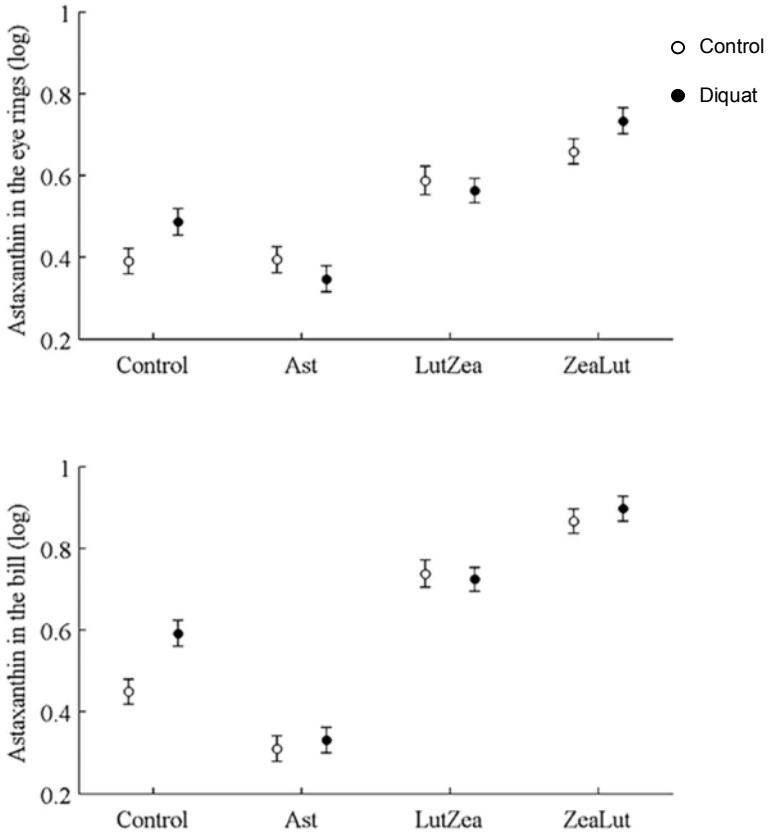

Figure 6. *Levels of astaxanthin in the eye rings and in the bill after diquat exposure depending on the carotenoid treatment. Least squared means ± se from the models (see Methods and Table 3).*

In contrast to astaxanthin, papilioerythrinone was unaffected by diquat (any trait: $P > 0.16$; Table 3). The best-fitted model (Table 4) always reported a significant CAR influence (all traits: $P < 0.010$; Fig. 4SM). In the eye rings, LutZea and ZeaLut birds did not differ ($P = 0.526$), but other comparisons were significant ($P < 0.012$). In the bill, all CAR groups differed ($P < 0.009$), with LutZea showing higher levels than ZeaLut, and Ast showing the lowest values. In the legs, LutZea presented higher papilioerythrinone levels than other groups (all P's < 0.017; differences among other groups $P > 0.13$; Fig. 4SM).

Tocopherol, but not retinol, was also detected in the ornaments. In the eye ring, the diquat × CAR interaction showed a trend toward significance ($P = 0.056$), with diquat decreasing tocopherol values in controls only ($P = 0.021$; Tables 3 and 2SM for raw data; see also Fig. 7). In the same model, the CAR factor ($P = 0.017$) showed that ZeaLut partridges had higher tocopherol levels than LutZea and control birds (both $P < 0.016$), but Ast birds also showed higher vitamin levels than LutZea and control animals (both $P < 0.039$; other comparisons $P > 0.75$).

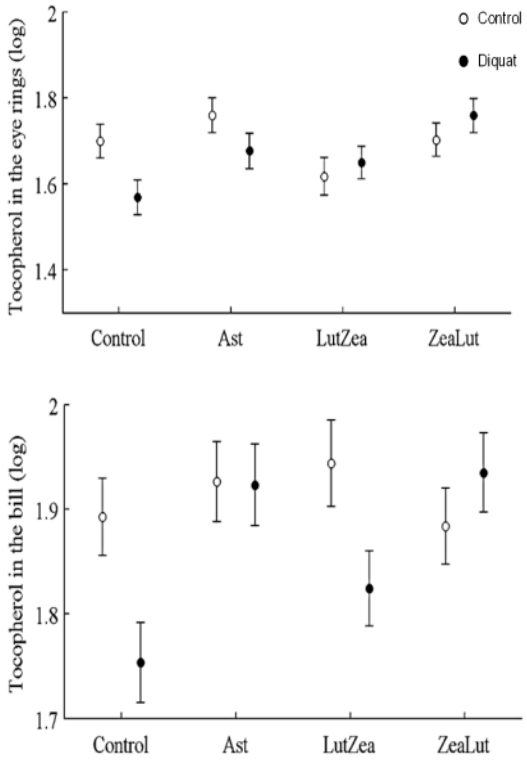

Figure 7. *Levels of tocopherol in the eye rings and bill after diquat exposure depending on the carotenoid treatment. Least squared means ± se were obtained from the models (see Methods and Table 3).*

In the bill, tocopherol was also affected by diquat × CAR (Table 3 and Fig. 7). Diquat decreased tocopherol values in control and LutZea individuals (both $P < 0.05$; Fig. 7). The CAR factor ($P = 0.035$) only indicated that controls had lower values than Ast and ZeaLut (both $P < 0.020$). Finally, only the CAR effect was significant in the legs (Tables 3 and 4). ZeaLut birds showed the highest tocopherol concentrations in the legs (both $P < 0.005$ when compared to LutZea and controls; $P = 0.085$ when compared to Ast).

3.3.2. Plasma and internal tissues

With regard to circulating carotenoids, lutein showed a significant diquat × CAR interaction (Table 3). Among CAR groups, only controls showed significantly higher lutein levels with diquat ($P = 0.039$; control: 0.98 ± 0.01; diquat: 1.02 ± 0.01, log-values; Fig. 8). In the case of zeaxanthin, although the CAR × diquat interaction was non-significant ($P = 0.200$; Table 3), the *post hoc* comparison within the control-CAR group showed a similar diquat effect ($P = 0.033$; control: 0.86 ± 0.02; diquat: 0.91 ± 0.02; Fig. 8).

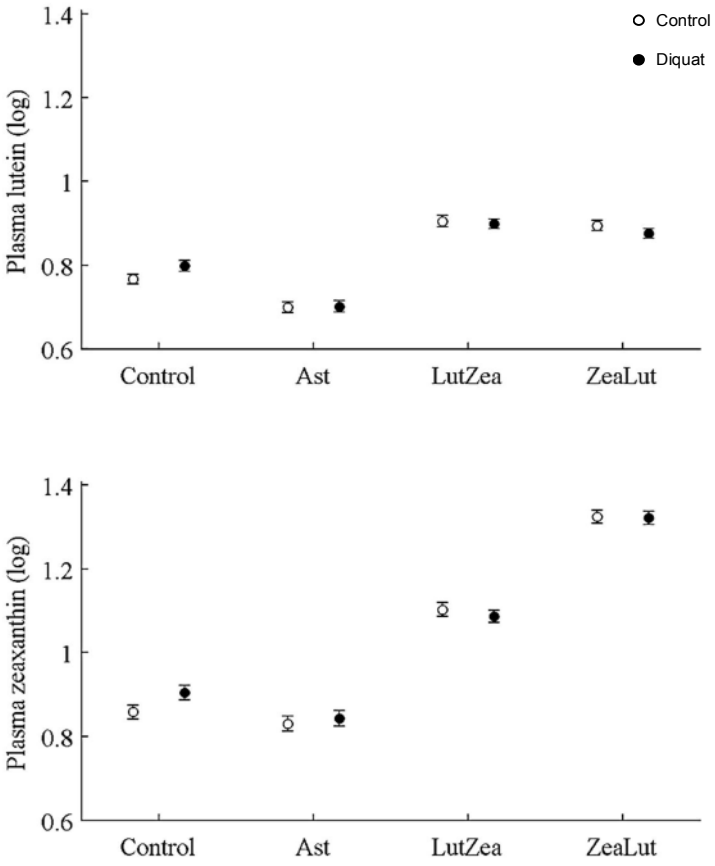

Figure 8. *Levels of lutein and zeaxanthin in plasma after diquat exposure depending on the carotenoid treatment. Least squared means ± se were obtained from the models (see Methods and Table 3).*

1

2 With regard to plasma vitamins, tocopherol was unaffected by the diquat × CAR
3 interaction (Table 3). Nonetheless, diquat showed a significant effect (Table 4), with
4 tocopherol values decreasing after the exposure (control: 1.08 ± 0.02; diquat: 1.03 ± 0.02).
5 No factor or interaction was significant in the case of plasma retinol (all $P > 0.10$; Table 4).

6 In the liver, the diquat × CAR interaction did not affect lutein levels (Table 3). The
7 best-fitted model reported a strong significant CAR effect (Table 4). LutZea and ZeaLut
8 birds did not differ ($P = 0.103$) and showed the highest lutein levels (Fig. 5SM). The other
9 comparisons always reported $P < 0.001$, and the Ast group showed the lowest value (Table
10 3SM). In contrast, liver zeaxanthin showed a significant CAR × diquat interaction (Table

3). This effect was mostly due to diquat reducing zeaxanthin levels in ZeaLut birds (P = 0.028), and a trend in the opposite direction among controls (P = 0.064; Fig. 9). Importantly, such as in the case of astaxanthin in ornaments, the CAR factor ($P < 0.001$) reported increasing liver zeaxanthin values in the following order: Ast, control, LutZea and ZeaLut (all comparisons: $P < 0.040$).

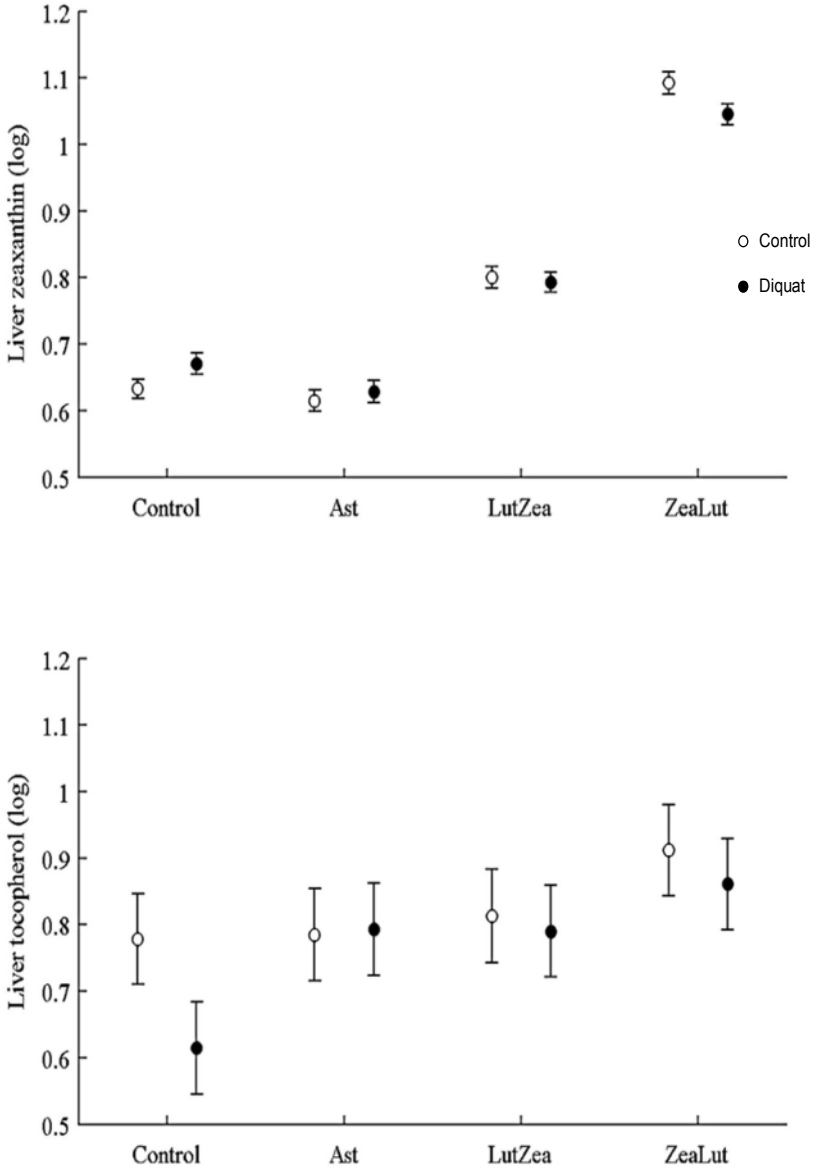

Figure 9. *Levels of zeaxanthin and tocopherol in the liver after diquat exposure depending on the carotenoid treatment. Least squared means ± se were obtained from the models (see Methods and table 3).*

With regard to liver vitamins, tocopherol was affected by CAR × diquat (Table 3). Among CAR groups, only control values showed a diquat effect on tocopherol, i.e. a decline ($P = 0.001$; Fig. 9). The CAR factor in the same model ($P < 0.001$) indicated significant differences following the order shown above for liver zeaxanthin (all $P < 0.003$), but here control and Ast birds did not differ ($P = 0.699$). In the case of liver retinol, both free and esterified retinol forms were detected, the two values being added for analyses (i.e. vitamin A). This variable was unaffected by CAR × diquat (Table 3) but showed a significant CAR effect (Table 4). LutZea and ZeaLut birds did not differ ($P = 0.133$), with Ast animals reporting the highest level, and control birds the lowest (other $P < 0.001$; Fig. 5SM).

In the subcutaneous fat, no carotenoid or vitamin was affected by CAR × diquat (all P values > 0.80; Table 3). The best-fitted models always reported a significant CAR effect (Table 4; Fig. 6SM; except for tocopherol). In the case of lutein, all groups differed from each other (all $P < 0.001$), except ZeaLut vs. control ($P = 0.915$). The LutZea group reported the highest lutein levels, and Ast birds the lowest. For zeaxanthin, LutZea birds tended to show higher values than controls ($P = 0.062$), with other groups significantly differing from each other (all $P < 0.012$). ZeaLut birds presented the highest zeaxanthin values, whereas Ast again showed the lowest. Tocopherol was not affected by any factor or interaction (all $P > 0.10$; Table 3; Fig. 7SM). With regard to retinol, all the groups differed from each other (CAR factor in Table 4), except ZeaLut and LutZea ($P = 0.955$). Ast and control birds showed the highest and lowest values, respectively (all $P < 0.001$; Fig. 6SM).

3.3.3. Oxidative stress biomarkers

PLAOX showed a three-way CAR × diquat × sex interaction (Table 3; Fig. 10). Diquat decreased hydrosoluble antioxidant levels in LutZea males ($P = 0.02$), showing a trend in the same direction in females, but in the ZeaLut group ($P = 0.06$; Fig. 10). No factor or interaction remained (all $P > 0.18$) when removing uric acid and albumin covariates (though they showed $P < 0.057$).

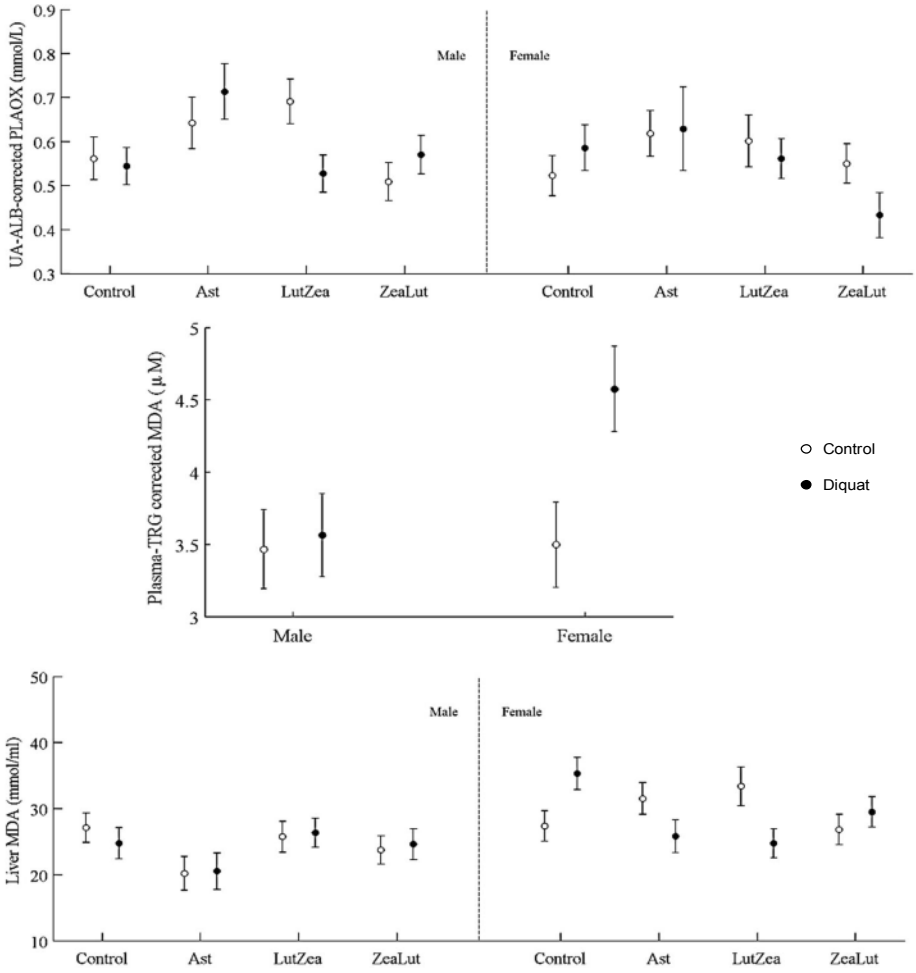

Figure 10. *Levels of oxidative stress biomarkers after diquat exposure depending on the carotenoid treatment. Least squared means ± se from the models (see Methods and Table 3).*

In plasma MDA, CAR × diquat was non-significant ($P = 0.466$; Table 3), but diquat × sex interacted (Table 4; Fig. 10). Diquat-treated females showed higher lipid peroxidation than control females ($P = 0.001$; males did not differ: $P = 0.752$). The interaction did not change ($P = 0.008$) when removing the triglyceride covariate. The CAR group was never significant ($P > 0.5$). In liver MDA, the three-way interaction again arose (Table 3; Fig. 10). Diquat increased MDA values in control females ($P = 0.009$), but decreased MDA in LutZea ($P = 0.014$) and Ast (but at $P = 0.079$) females. Moreover, diquat control-CAR females also tended to endure higher liver MDA values than diquat ZeaLut females ($P = 0.068$). No difference was found in males (all $P > 0.10$). The CAR group in the model was

not significant (P = 0.289). No factor or interaction reported significant terms in heart MDA (Table 4; all P > 0.12).

Finally, in the case of erythrocyte resistance to oxidative stress, the CAR × diquat interaction only showed a weak trend toward significance (P = 0.090; Table 3), but the best-fitted model reported a significant diquat effect (Table 4; Fig. 11). The CAR factor was not significant (all P > 0.50).

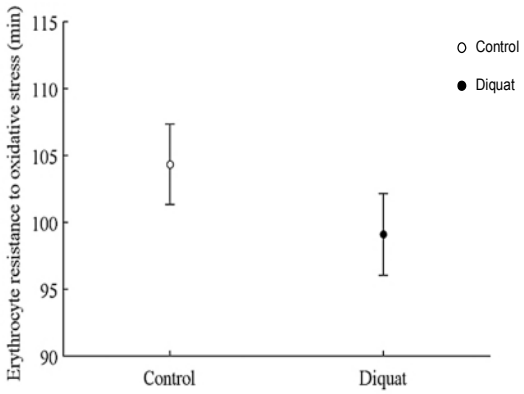

Figure 11. *Effect of the diquat treatment on the erythrocyte resistance to oxidative stress. Least squared means ± se from the models (see Methods and Table 4).*

4. Discussion

Our results as a whole suggest that the availability of certain carotenoids in the diet and the level of oxidative stress can interact to produce pigmentation in avian ornaments. As predicted, a higher dietary content of lutein vs. zeaxanthin (LutZea) led to a higher papilioerythrinone accumulation in the red ornaments, whereas the opposite (ZeaLut) led to a higher astaxanthin deposition. Birds fed with higher zeaxanthin and lutein proportions showed the reddest ornaments, but the first (ZeaLut) showed the reddest traits (eye rings and legs) at the end of the study. Furthermore, the oxidative challenge produced redder bills and higher astaxanthin deposition in the bare parts of some birds, the latter depending on tocopherol levels in the same tissue.

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4.1. Covariation between vitamins and carotenoids

The carotenoid treatments affected tocopherol levels in tissues. However, this only partially agreed with diet composition, which showed the highest vitamin values in the ZeaLut and, particularly, the Ast groups (Table 1). In legs, plasma and liver, only ZeaLut birds showed higher tocopherol levels than other groups. The lack of high tocopherol values in Ast partridges in these tissues could be explained by astaxanthin interfering with vitamin absorption (e.g. Giraudeau et al. 2013; also below). Nonetheless, both Ast and ZeaLut groups showed the highest tocopherol levels in the other ornaments. In the case of retinoids, Ast birds also showed the highest value in liver and fat, but ZeaLut and LutZea groups did not differ.

Results also suggest that carotenoids protected vitamin E from oxidative stress. In the bill, eye rings, and liver, diquat decreased tocopherol levels, but only among birds that did not receive carotenoid supplements. This supports the idea of mutual recycling and protective roles between tocopherol and carotenoids (Mortensen et al. 2001; Catoni et al. 2008; Surai et al. 2012). The only exception was the diquat-mediated reduction in tocopherol levels in the bill of LutZea birds. Regardless, we must consider that, among carotenoids, lutein (i.e. the most abundant carotenoid in LutZea birds) is the weakest antioxidant (Britton 1995; Martínez et al. 2008; see also below).

To discriminate carotenoid effects from the influence of vitamin variability in the diet, all the statistical models were controlled for tocopherol and retinoid levels in the tissues. The problem of collateral variation of antioxidants in a supplemented diet has mostly been ignored in experiments aiming to strictly manipulate dietary carotenoid levels. For instance, Stirnemann et al. (2009) and Toomey & McGraw (2011, 2012) have used the same beadlets including zeaxanthin and tocopherol, but vitamin levels were not considered in their analyses. The addition of other antioxidants as excipients in carotenoid supplements would protect carotenoids during storage. In the other direction, the addition of carotenoids to the pelleted food could also have protected antioxidant vitamins in the basal diet, thus disrupting the original covariation among the levels of different compounds (Table 2; Catoni et al. 2008). In any event, we must note that diquat effects within a particular carotenoid treatment were independent of vitamin variability in that group as both diquat

and control birds should have received the same vitamin amounts. In summary, results must be carefully interpreted in the light of vitamin covariation.

4.2. Metabolic pathway of dietary carotenoids

We predicted that birds supplemented with astaxanthin should produce the most pigmented ornaments as biotransformation is not required. Surprisingly, dietary astaxanthin was apparently not absorbed. It was not detected in blood and other internal tissues. Moreover, astaxanthin seems to have interfered with lutein, zeaxanthin and tocopherol acquisition, as the circulating levels of these molecules declined in Ast birds. Consequently, ketocarotenoid deposition in ornaments and trait redness were reduced. Carotenoid competition during intestinal absorption and/or incorporation into the chylomicrons (e.g. Tyssandier et al. 2002; Canene-Adams and Erdman Jr. 2009) can be argued considering the literature on humans (reviewed in Furr and Clark, 1997; van der Berg 1999). In birds, competitive interactions of beta-carotene vs. lutein or zeaxanthin during intestinal absorption have also been reported for poultry diets (Wang et al. 2010). Interestingly, in the opposite direction, flamingoes (*Phoenicopterus ruber*) fed with lutein or zeaxanthin were unable to absorb these two pigments, but were 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). Our partridges also differ from European storks (*Ciconia ciconia*) naturally feeding on crayfish (*Procambarus clarkii*) containing high astaxanthin concentrations because they showed redder skin and higher astaxanthin concentrations in blood than controls (Negro and Garrido-Fernández, 2000). Phylogenetic differences may explain this. Astaxanthin is common in waterbirds feeding on fishes and aquatic invertebrates (an important astaxanthin source), but not among other avian species (McGraw 2006). The red-legged partridge is a terrestrial granivorous gallinacean, and thus astaxanthin is probably infrequent in their natural diet. For this reason, the capacity for assimilating astaxanthin may not have evolved.

On the other hand, our manipulation mostly supports the biotransformation pathway proposed for red-legged partridge carotenoids (i.e. García de Blas et al. 2014); that is, lutein acting as the main papilioerythrinone precursor, with zeaxanthin acting as the main

astaxanthin substrate. Lutein and zeaxanthin levels rose in the blood, liver and fat according to their relative abundance in the diet. Similarly, papilioerythrinone and astaxanthin in ornaments increased in higher amounts in LutZea and ZeaLut groups, respectively. The results support previous correlative findings in the same species (García de Blas et al. 2015) and demonstrate that ketocarotenoids giving color to red-legged partridge ornaments are influenced by the availability of the most common hydroxycarotenoids in birds (McGraw 2006). As previously mentioned, lutein and zeaxanthin are the most frequently described and abundant carotenoids in the food and blood of many bird species, as well as the most common substrates for red ketocarotenoids in ornaments, at least among non-aquatic species (Surai et al. 2001; McGraw 2006). In passerines, lutein levels always prevail over zeaxanthin levels in both blood and diet, commonly at a 70:30 ratio (lutein:zeaxanthin) or higher (e.g. McGraw et al. 2004), which could also reflect the dietary content (McGraw 2006). Our manipulation supports this for a gallinacean species. Moreover, McGraw et al. (2004) proposed that birds should prioritize zeaxanthin accumulation because this pigment would proportionally contribute more to coloring red ornaments compared to lutein. This has only been supported by correlations between the ratio of these two principal hydroxycarotenoids in the body and the ratio of pigments deposited in the ornaments (McGraw and Gregory 2004; García de Blas et al. 2015). Our experimental results also confirm this, and support, to some extent, the hypothesis that carotenoid-based signaling reveals an individual's capacity to find specific carotenoids in the environment (i.e. Endler 1980).

Finally, the fact that astaxanthin and papilioerythrinone were only found in bare parts validates our previous findings (Garcia de Blas et al. 2015) and again supports the idea that biotransformation takes place *in situ*, at the colored trait, something only explored and described in passerines (McGraw 2004, 2009 for eleven species; but see Del Val et al. 2009 and McGraw and Toomey 2010 for two other passerine species).

4.3. Dietary hydroxycarotenoids contributing to color

Lutein and zeaxanthin supplementation attenuated the color decline observed throughout the breeding season in red-legged partridges (Alonso-Alvarez et al. 2008). Consistently

with the highest rate of astaxanthin deposition in the ornaments, the ZeaLut treatment produced the reddest birds at the end of the study. We must note that statistical analyses testing the CAR effect only (Table 2) did not include data from birds treated with diquat at the last sampling event, which reduced the sample size by half. When color was tested by controlling the diquat effect, differences between the ZeaLut and LutZea group arose (Tables 3 and 4). The fact that ZeaLut birds were the reddest supports the view that individuals should try to obtain the highest zeaxanthin amounts in the diet to generate ornaments with the highest astaxanthin levels (see also García de Blas et al. 2015). The coexistence of astaxanthin and papilioerythrinone in the same ornaments could nonetheless be explained by the abundance of lutein in the diet and the contribution of papilioerythrinone to color (García de Blas et al. 2013, 2014). Astaxanthin is the most conjugated carotenoid, and hence, the reddest (and most abundant) pigment in red-legged partridge ornaments. However, it has been shown that variability in papilioerythrinone levels in the red head traits can contribute to explaining color variation, at least in a correlational sample of these birds (i.e. García de Blas et al. 2013).

4.4. Oxidative stress and carotenoids

Results support that diquat indeed increased oxidative stress in our birds. Diquat is a free radical generator previously used in the same dose and species, which reported effects on the antioxidant machinery (Alonso-Alvarez et al. 2009). Partridges treated with diquat showed weaker erythrocyte resistance to hemolysis when blood was exposed to another free radical source (AAPH; e.g. Alonso-Alvarez et al. 2006). The impact on circulating hydrosoluble antioxidants (PLAOX) was less evident, showing declines in some carotenoid groups only, but depending on the sex. Diquat-treated females, but not males, also showed higher levels of plasma lipid peroxidation (a marker of oxidative damage). Female partridges could be more sensitive to oxidative damage during reproduction perhaps due to the costs associated with egg production and antioxidant allocation to eggs (e.g. Williams 2005). Accordingly, female red-legged partridges producing eggs with higher hatching success endured higher lipid peroxidation in erythrocytes (i.e. Alonso-Alvarez et al. 2010). Similarly, diquat-treated females, but not males, showed higher lipid peroxidation in the

liver than controls, but only among birds that did not receive carotenoid supplements. In fact, LutZea and Ast females treated with diquat showed a decline in liver MDA values compared to controls of the same group (Fig. 10). Therefore, liver MDA findings support the antioxidant role of xanthophylls involved in coloration, at least for females. This role has been questioned repeatedly, at least for avian species (Hartley & Kennedy 2004; Costantini & Møller 2008; Isaksson & Andersson 2008; but see Simons et al. 2012). Results from other oxidative stress markers are, however, less consistent.

Higher PLAOX levels in Ast and LutZea birds of both sexes compared to controls were found (see Fig. 3; the last at $P = 0.052$). However, PLAOX did not increase in ZeaLut partridges. The antioxidant potential of each pigment is linked to the number of conjugated double bonds: 13, 11 and 10 for astaxanthin, zeaxanthin, and lutein, respectively (Britton 1995, 2009; Martínez et al. 2008). Therefore, an increase in PLAOX among ZeaLut birds is predictable. However, we must consider that PLAOX mostly assesses the presence of hydro-, but not lipid-, soluble antioxidants (Miller et al. 1993; Cohen et al. 2007). Thus, a higher PLAOX may also be due to the mobilization of other antioxidants (e.g. vitamin C) to fight off a challenge of some type (a hormetic effect; Costantini et al. 2010). This view particularly agrees with the highest PLAOX values in Ast birds. These animals did not show astaxanthin in plasma and even experienced lower plasma lutein, zeaxanthin and tocopherol levels than controls (above). Similarly, Ast birds did not show astaxanthin in the liver, but accumulated large amounts of vitamin A in this organ, perhaps to protect the liver from some toxic insult (García de Blas et al. 2015). Nonetheless, we found only one study supporting this, in which rats fed with astaxanthin endured an impairment of the liver enzymes involved in detoxification (Ohno et al. 2011). In summary, if PLAOX did not exclusively reveal the antioxidant capacity of circulating carotenoids, the lack of higher PLAOX values in ZeaLut birds could merely be due to other antioxidants being unaltered. The conclusion is that the antioxidant role of carotenoid cannot easily be demonstrated from PLAOX measures.

4.5. Oxidative stress and carotenoid biotransformation

Although the proximate cost of ketocarotenoid-based signaling in red-legged birds may, at least partially, involve increased foraging effort to obtain large zeaxanthin amounts in the

diet, the requirement of biotransformation to produce red traits provides another substrate for natural selection. Birds exposed to diquat generated redder bills, which contradicts the constraining impact of oxidative stress on health (e.g. Monaghan et al. 2009; Dowling & Simmons 2009; Costantini 2014). In contrast, red-legged partridges exposed to the same diquat dose and duration in another experiment, but during the first weeks of life, produced paler red colors in adulthood (Alonso-Alvarez & Galván 2011). We must nonetheless consider that adverse conditions during early periods of life are particularly damaging (Metcalfe & Monaghan 2001). Young individuals may not have fully developed antioxidant machinery (Metcalfe & Alonso-Alvarez 2010) to properly manage such an oxidative challenge. Pigment levels in partridge ornaments seem to support the change in color and revealed that carotenoid concentrations also increased under diquat exposure. Interestingly, the increase in these tissues was detected for astaxanthin, but not papilioerythrinone.

Astaxanthin production from its substrate requires two oxidation steps, whereas papilioerythrinone would require only one oxidation plus a hydrogenation (McGraw et al. 2006; LaFountain et al. 2013, García-de Blas et al, 2014). A large availability of free radicals derived from diquat activity could have favored hydroxycarotenoid biotransformation rates, which should be clearer in the case of astaxanthin. This could have taken place by increasing the enzyme (oxygenase, also named ketolase) gene expression or the enzymatic activity by favoring the cofactors of the reaction. Although the characterization of this oxygenase has not been fully accomplished, it requires the presence of Fe^{2+} and their activity is oxygen-dependent (Choi et al. 2007; Makino et al. 2008).

Biotransformation seems to be higher among birds with the highest availability of the main ketocarotenoid precursor; that is, ZeaLut birds (see in the eye ring; though $P = 0.057$; Fig. 6). However, the clearest effect was found in diquat-treated birds that did not receive any carotenoid supplementation (Fig. 6). The effect in these two CAR groups would agree with bill color findings (though the interaction was non-significant). The effect on non-supplemented birds could be due to better zeaxanthin availability in blood (Fig. 8) and liver (Fig 9) in this group. Higher circulating levels of zeaxanthin could be a consequence of an active mobilization from stores (liver) and/or better intestinal absorption, both for combating oxidative stress (e.g. Alonso-Alvarez et al. 2008; McClean et al. 2011;

but see Isaksson & Andersson 2008). Recent works suggest that xanthophyll absorption in the intestinal mucosa can be actively regulated by specific protein scavenger receptors such as the class B member 1 (SR-B1; Hill & Johnson 2012; Sato et al. 2012). How diquat may have favored receptor activity can only be speculated. In addition to the direct effects derived from increased superoxide production (damage on main biomolecules; e.g. Jones & Vale 2000), diquat may interfere in redox signaling mechanisms whose derived effects are still poorly understood (see Cristovao et al. 2009 for the effect of a very similar bipyridyl compound, paraquat, on redox signaling enzymes). Nonetheless, we must note that redox signaling disruption is also considered a component of oxidative stress (Jones 2006; Sohal & Orr 2012).

In any event, in order to test whether higher astaxanthin levels in ornaments are due to higher zeaxanthin availability in the body (i.e., not to higher biotransformation rates), we also added plasma or liver zeaxanthin levels as covariates in models testing bill and eye ring astaxanthin concentrations. As expected, a positive link between ornament astaxanthin and plasma zeaxanthin values was observed (also García de Blas et al. 2015), but this did not change the CAR × diquat interaction or *post hoc* tests (always $P < 0.05$). Moreover, diquat did not increase zeaxanthin values in internal tissues in the other group showing increased astaxanthin deposition in ornaments (ZeaLut; Fig. 8). Nonetheless, some results may still support the availability of carotenoid precursors as a key factor favoring biotransformation. Diquat decreased tocopherol values in ornaments among non-supplemented birds (Fig. 7). When tocopherol levels in bare parts is not statistically controlled for as a covariate, differences in astaxanthin levels among the same control birds (Fig. 6) disappear (both traits: $P > 0.60$), but not among ZeaLut birds (becoming significant at $P = 0.036$). The result suggests that biotransformation is stimulated by oxidative stress when the amount of carotenoid precursors in the diet surpasses some threshold. When this is not the case, color is not impaired but tocopherol levels are consumed to control the challenge.

In summary, the overall results suggest that specific carotenoid precursors must be sufficiently available and that oxidative status must be well-adjusted in order to produce the most pigmented red ornaments. In agreement with this, redder integuments have also been

observed in red-legged partridges exposed to other chemicals (i.e. pesticides and heavy metals) that induce oxidative stress (López-Antía et al. 2015a,b; Vallverdú-Coll et al., 2015). The findings support the view that oxidative stress is not only a constraint for the expression of optimal phenotypes, but that low levels are necessary for many functions (Jones 2006; Metcalfe & Alonso-Alvarez 2010; Isaksson et al. 2011). Furthermore, the study supports recent claims from Hill and Johnson (2012 and Johnson and Hill 2013) hypothesizing that carotenoid-based traits could be signaling an individual's efficiency to manage oxidative stress. The results also validate the older work of Völker (1957), suggesting that good oxidative metabolism is necessary to biotransform carotenoids involved in red coloration. However, in contrast to the works of Hill and Johnson, our experiment also highlights the importance of resource allocation trade-offs because the level of some carotenoids in the body apparently influence the role of oxidative stress in biotransformation. Finally, we cannot conclude this discussion without applying a life-history perspective. We argue that high levels of sexual signaling under high oxidative stress could constitute a sort of terminal investment, with individuals increasing their chances of reproducing when their perception of future survival becomes negative (Velando et al. 2007; Romero-Haro & Alonso-Alvarez 2015).

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References

Alonso-Alvarez C., Bertrand S., Devevey G., Gaillard M., Prost J., Faivre B., Sorci G. (2004) An experimental test of the dose-dependent effect of carotenoids and immune

activation on sexual signals and antioxidant activity. The American Naturalist 164:651-659.

Alonso-Alvarez C., Bertrand S., Devevey G., Prost J., Faivre B., Chastel O., Sorci G. (2006) An experimental manipulation of life-history trajectories and resistance to oxidative stress. Evolution, 60(9):1913–1924.

Alonso-Alvarez C., Pérez-Rodríguez L., Mateo R., Chastel O., Viñuela J. (2008) The oxidation handicap hypothesis and the carotenoid allocation trade-off. Journal of Evolutionary Biology, 21:1789–1797.

Alonso-Alvarez C., Pérez-Rodríguez L., Garcia J.T., Viñuela, J. (2009) Testosterone-mediated trade-offs in the old age: A new approach to the immunocompetence handicap and carotenoid-based sexual signalling. Proceedings of the Royal Society B 276:2093-2101.

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

Alonso-Alvarez C., Galván I. (2011) Free radical exposure during development 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., Ferrero M.E., Garcia-de Blas E., Casas F., Mougeot F. (2012) Adjustment of female reproductive investment according to male carotenoid-based ornamentation in a gallinaceous bird. Behavioral Ecology and Sociobiology 66:731–742.

Andersson M. (1994) Sexual selection. Princeton University Press, New Jersey.

Blache D., Prost M. (1992) Free radical attack: biological test for human resistance capability. A Lunar-Based Chemical Analysis Laboratory (eds C.Ponnamperuma & C.W.Gehrke), pp. 82–98. Deepak A, Hampton, VA.

Blem C.R. (2000) Energy Balance. In: G. Causey Whittow (Ed), Sturkie's Avian physiology. Fifth edition, Academic Press, San Diego, California, USA, pp 327-341.

Blount J.D., Metcalfe N.B., Birkhead T.R, Surai P.F. (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. Science 300:125-127.

Bradbury J.W., Vehrenkamp S.L. (1998) Principles of animal communication. Sunderland, MA: Sinauer.

Britton G. (1995) Structure and properties of carotenoids in relation to function. The FASEB Journal. 1551-1558.

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

Brush A.H., Power D.M. (1976) House finch pigmentation: Carotenoid metabolism and effect of diet. The Auk 93:725–739.

Brush A.H. (1990) Metabolism of carotenoid pigments in birds. The FASEB Journal 4:2969–2977.

Burton G.W. (1989) Antioxidant action of carotenoids. Journal of Nutrition 119(1):109-111.

Canene-Adams K., Erdman Jr J.W. (2009) Absorption, transport, distribution in tissues and bioavailability in Carotenoids vol 5 Nutrition and Health, Pp:115-148.

Catoni C., Peters A., Schaefer M. (2008) Life-history trade-offs are influenced by the diversity, availability and interactions of dietary antioxidants. Animal Behaviour 76:1107–1119.

Choi S.K., Harada H Matsuda S., Misawa N. (2007) Characterization of two β-carotene ketolases, CrtO and CrtW, by complementation analysis in *Escherichia coli*. Applied Microbiology and Biotechnology 75:1335-1341.

Cohen A., Klasing K., Ricklefs R. (2007) Measuring circulating antioxidants in wild birds. Comparative Biochemistry and Physiology B 147, 110–121.

Costantini D., Møller A.P. (2008) Carotenoids are minor antioxidants for birds. Functional Ecology 22:367–370.

Costantini D., Metcalfe N.B., Monaghan P. (2010) Ecological processes in a hormetic framework. Ecology Letters. 13:1435-1447.

Costantini D. (2014) Oxidative Stress and Hormesis in Evolutionary Ecology and Physiology. A Marriage Between Mechanistic and Evolutionary Approaches. Springer.

Cristóvão AC., Choi D.H., Baltazar G., Beal M.F., Kimcorresponding Y.-S. (2009) The role of NADPH oxidase 1–derived reactive oxygen species in paraquat-mediated dopaminergic cell death. Antioxid Redox Signal. 2009 Sep; 11(9): 2105–2118.

Darwin C. (1871) The Descent of Man and Selection in Relation to Sex, John Murray, London.

Dowling D.K., Simmons L.W. (2009) Reactive oxygen species as universal constraints in life-history evolution. Proceedings of the Royal Society B 276:1737–1745.

del Val E., Senar J.C., Garrido-Fernández J., Jarén M., Borràs A., Cabrera J., Negro, J.J. (2009a). The liver but not the skin is the site for conversion of a red carotenoid in a passerine bird. Naturwissenschaften 96:797-801.

del Val E., Senar J.C., Garrido-Fernández J., Jarén M., Borràs A., Cabrera J., Negro, J.J. (2009b). Hepatic conversion of red carotenoids in passerine birds. Naturwissenschaften 96:989–991.

El-Agamey A., McGarvey D.J. (2008) Carotenoid Radicals and Radical Ions, in Carotenoids Volume 4: Natural Functions Ed Britton G., Liaaen-Jensen S., Pfander H. Birkhäuser Verlag.

Endler J.A. (1980) Natural selection on color patterns in *Poecilia reticulata*. Evolution 34: 76–91.

Endler J.A. (1983) Natural and sexual selection on colour patterns in poeciliid fishes. Environmental Biology of Fishes 9: 173–190.

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

Fox D.L., McBeth J.W. (1970) Some dietary carotenoids and blood carotenoid levels in flamingos. Comparative Biochemistry and Physiology 34:707–713.

Furr H.C., Clark R.M. (1997) Intestinal absorption and tissue distribution of carotenoids. Nutritional Biochemistry 8:364-377.

Galvan I., Alonso-Alvarez C. (2009) The expression of melanin-based plumage is separately modulated by exogenous oxidative stress and a melanocortin. Proceedings of the Royal Society B 276:3089–3097.

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

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

García-de Blas, E., Mateo, R., Guzmán Bernardo, F.J., Rodríguez Martín-Doimeadios, R.C., 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., 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.

Getty T. (2006). Sexually selected signals are not similar to sports handicaps. Trends in Ecology & Evolution 21: 83–88.

Girard A., Madani S., El Boustani E.S., Belleville J., Prost J. (2005) Changes in lipid metabolism and antioxidant defense status in spontaneously hypertensive rats and Wistar rats fed a diet enriched with fructose and saturated fatty acids. Nutrition 21:240–248.

Giraudeau M., Sweazea K., Butler M.W., McGraw K.J. (2013) Effects of carotenoid and vitamin E supplementation on oxidative stress and plumage coloration in house finches (*Haemorhous mexicanus*) Comparative Biochemistry and Physiology A 166:406–413.

Godin J.G.J., McDonough H.E. (2003) Predator preference for brightly colored males in the guppy: a viability cost for a sexually selected trait. Behavioral Ecology 14:194–200

Grafen A. (1990) Biological signals as handicaps. Journal of Theoretical Biology 144:517-546.

Griffiths R., Double M.C., Orr K., Dawson R.J.G. (1998) A DNA test to sex most birds. Molecular Ecology 7:1071–1075.

Halliwell B., Gutteridge J.M.C. (2007) Free Radicals in Biology and Medicine. Oxford: Oxford University Press.

Hasson O. (1997) Towards a general model of biological signaling. Journal of Theoretical Biology, 185:139-156.

Hata M., Hata M. (1972) Carotenoid pigments in goldfish. V. Conversion of zeaxanthin to astaxanthin Bulletin of the Japanese Society of Fisheries Science 38:339-343

Hill G.E. (1990) Female house finches prefer colorful males: Sexual selection for a condition-dependent trait. Animal Behaviour 40:563–570.

Hill G.E. (2006) Environmental regulation of ornamental coloration In: Bird Coloration I: Mechanisms and measurements (Hill, G. E. & McGraw, K. J. eds.). Harvard University Press, Cambridge, MA.

Hill G.E., Johnson J.D. (2012) The vitamin A–redox hypothesis: a biochemical basis for honest signaling via carotenoid pigmentation. American Naturalist 180:E127–E150.

Hörak P., Saks L., Zilmer M., Karu U., Zilmer K. (2007) Do dietary antioxidants alleviate the cost of immune activation? An experiment with greenfinches. American Naturalist 170:625–635.

Isaksson C., Andersson S. (2008) Oxidative stress does not influence carotenoid mobilization and plumage pigmentation. Proceedings of the Royal Society B 275:309–314.

Isaksson C., Sheldon B.C., Uller T. (2011) The challenges of integrating oxidative stress into life history biology. Bioscience. 60:194-202.

Johnson J.D., Hill G.E. (2013) Is carotenoid ornamentation linked to the inner mitochondrial membrane potential? A hypothesis for the maintenance of signal honesty. Biochimie 95:436–444.

Jones D.P. (2006) Redefining oxidative stress. Antioxidants & Redox Signaling. 8:1865-1879.

Jones G.M., Vale J.A. (2000) Mechanisms of toxicity, clinical features, and management of diquat poisoning: a review. Journal of Toxicology - Clinical Toxicology 38:123-128.

Kodric-Brown A. (1985) Female preference and sexual selection for male coloration in the guppy (*Poecilia reticulata*). Behavioral Ecology and Sociobiology 17:199–205.

LaFountain A.M., Frank H.A., Prum R.O. (2013) Carotenoids from crimson and maroon plumages of Old World orioles (Oriolidae). Archives of Biochemistry and Biophysics 539:126-132.

Lesgards J.F., Durand P., Lassarre M., Stocker P., Lesgards G., Lanteaume A., Prost M., Lehucher-Michel M.P. (2002) Assessment of lifestyle effects on the overall antioxidant capacity of healthy subjects. Environmental Health Perspectives 110:479–487.

Littell R.C., Milliken G.A., Stroup W.W., Wolfinger R.D., Schabenberger O. (2006) SAS for mixed models. 2nd ed. SAS Institute, Cary, NC.

Lopez-Antia, A., Ortiz-Santaliestra, M.E., García-de Blas, E., Camarero, P.R., Mougeot, F., Mateo, R. (2015a). Adverse effects of thiram treated seed ingestion on the reproductive performance and the offspring immune function of the red-legged partridge. Environmental Toxicology and Chemistry 34:1320-1329.

Lopez-Antia, A., Ortiz-Santaliestra, M.E., Mougeot, F., Mateo, R. (2015b). Imidacloprid-treated seed ingestion has lethal effect on adult partridges and reduces both breeding investment and offspring immunity. Environmental Research 136: 97-107.

Lozano G. A. (1994) Carotenoids, parasites, and sexual selection. Oikos 70: 309-311.

Makino T., Harada H., Ikenaga H., Matsuda S., Takaichi S., Shindo K., Sandmann G., Ogata T., Misawa N. (2008) Characterization of cyanobacterial carotenoid ketolase CrtW and hydroxylase CrtR by complementation analysis in *Escherichia coli*. Plant and Cell Physiology 49:1867–1878.

Martínez A., Rodríguez-Gironés M.A., Barbosa A., Costas M. (2008) Donator acceptor map for carotenoids, melatonin and vitamins. The Journal of Physical Chemistry A 112:9037–9042.

Maynard Smith J. Harper D. (2003) Animal Signals. Oxford University Press.

Metcalfe N.B., Alonso-Alvarez C. (2010) Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Functional Ecology 24:984–996.

McGraw K.J. (2004) Colorful songbirds metabolize carotenoids at the integument. Journal of Avian Biology 35:471–476.

McGraw K.J., Gregory A.J. (2004) Carotenoid pigments in male American goldfinches: what is the optimal biochemical strategy for becoming colourful? Biological Journal of the Linnean Society. 83:273–280.

McGraw K.J., Hill G.E., Navara K.J., Parker R.S. (2004) Differential accumulation and pigmenting ability of dietary carotenoids in colorful finches. Physiological and Biochemical Zoology 77:484–491.

McGraw K.J. (2006) Mechanics of carotenoid-based coloration. In: Bird Coloration I: Mechanisms and measurements (Hill, G. E. & McGraw, K. J. eds.). Harvard University Press, Cambridge, MA.

McGraw K.J. (2009) Identifying anatomical sites of carotenoid metabolism in birds. Naturwissenschaften 96:987–988.

McClean C.M., Clegg M., Shafat A., Murphy M.H., Trinick T., Duly E., McLaughlin J., Fogarty M., Davison G.W. (2011) The impact of acute moderate intensity exercise on arterial regional stiffness, lipid peroxidation, and antioxidant status in healthy males. Research in Sports Medicine 19:1-13.

Metcalfe N.B., Monaghan P., et al.(2001) Compensation for a bad start: grow now, pay later? TRENDS in Ecology & Evolution.16:254-260.

Miller N.J., Sampson J., Candeias L.P., Bramley P.M., Rice-Evans C.A. (1996) Antioxidant activities of carotenes and xanthophylls. FEBS Letter 384:240–242.

Møller A.P., Biard C., Blount J.D., Houston D.C., Ninni P., Saino N., Surai P.F. (2000) Carotenoid-dependent signals: Indicators of foraging efficiency, immunocompetence or detoxification ability? Avian and Poultry Biology Reviews 11:137–159.

Monaghan P., Metcalfe N.B., Torres R. (2009) Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements, and interpretation. Ecology Letters 12:75–92.

Mortensen A., Skibsted L.H., Truscott T.G. (2001) The interaction of dietary carotenoids with radical species Archives of Biochemistry and Biophysics 385:13–19.

Mougeot F., Pérez-Rodríguez L., Sumozas N., Terraube J. (2009) Parasites, condition, immune responsiveness and carotenoid-based ornamentation in male red-legged partridge *Alectoris rufa*. Journal of Avian Biology 40:67-74.

Negro J.J., Garrido-Fernandez J. (2000) Astaxanthin is the major carotenoid in tissues of White storks (*Ciconia ciconia*) feeding on introduced crayfish (*Procambarus clarkii*). Comparative Biochemistry and Physiology B 126:347-352.

Ohkubo M., Tsushima M., Maoka T., et al.(1999) Carotenoids and their metabolism in the goldfish *Carassius auratus* (Hibuna). Comparative Biochemistry and Physiology B 124:333-340.

Ohno M., Darwish W.S., Ikenaka Y., Miki W., Ishizuka M. (2011) Astaxanthin can alter CYP1A-dependent activities via two different mechanisms: induction of protein expression and inhibition of NADPH P450 reductase dependent electron transfer. Food and chemical toxicology. 49:1285-1291.

Panfili G., Fratianni A., Irano M. (2004) Improved normal-phase high-performance liquid chromatography procedure for the determination of carotenoids in cereals. Journal of Agricultural and Food Chemistry 52: 6373-6377.

Pérez-Rodríguez L. (2008) Carotenoid-based ornamentation as a dynamic but consistent individual trait. Behavioral Ecology and Sociobiology 62:995-1005.

Pérez-Rodríguez L., Viñuela J. (2008) Carotenoid-based bill and eye ring coloration as honest signals of condition: an experimental test in the red-legged-partridge (*Alectoris rufa*). Naturwissenschaften 95:821–830.

Pérez-Rodríguez L., Mougeot F., Alonso-Alvarez C., Blas J., Viñuela J., Bortolotti G.R. (2008) Cell-mediated immune activation rapidly decreases plasma carotenoids but does not affect oxidative stress in red-legged partridges (*Alectoris rufa*). The Journal of Experimental Biology 211:2155–2161.

Pérez-Rodríguez L. (2009) Carotenoids in evolutionary ecology: re-evaluating the antioxidant role. BioEssays 31:1116–1126.

Pérez-Rodríguez L., Mougeot F., Alonso-Alvarez C. (2010) Carotenoid-based coloration predicts resistance to oxidative damage during an immune challenge. Journal of Experimental Biology 213:1685-1690.

Pietsch W. (2005) Agglomeration in Industry, Vol 1. Wiley-VCH-Verlag.

Rodríguez-Estival J., Martínez-Haro M., Martín-Hernando M.P., 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. Environmental Research 110:169-175.

Rojas Wahl R.U., Liansheng Z., Madison S.A., DePinto R.L., Shay B.J. (1998) Mechanistic studies on the decomposition of water soluble azo-radical-initiators. Journal of the Chemical Society, Perkin Transactions 2:2009–2018.

Romero-Haro A., Alonso-Alvarez C. (2014) Covariation in oxidative stress markers in the blood of nestling and adult birds. Physiological and Biochemical Zoology 87:353-362.

Romero-Haro A., Canelo, T., Alonso-Alvarez C. (2015) Early development conditions and the oxidative cost of social context in adulthood: an experimental study in birds. Frontiers in Ecology and Evolution 3:35. doi: 10.3389/fevo.2015.00035

Romero-Haro A., Alonso-Alvarez C. (2015) The level of an intracellular antioxidant during development determines the adult phenotype in a bird species: a potential organizer role for glutathione. American Naturalist 185:390–405.

Saks L., Mcgraw K.J., Hõrak P. (2003) How feather colour reflects its carotenoid content. Functional Ecology 17:555–561

SAS Institute (2001) SAS/STAT software: changes and enhancements, version 8.2. SAS, Cary, NC.

SatoY., Suzuki R., Kobayashi M., Itagaki S., Hirano T., Noda T., Mizuno S., Sugawara M., Iseki, K. (2012) Involvement of cholesterol membrane transporter niemann-pick c1-like 1 in the intestinal absorption of lutein. Journal of Pharmacy and Pharmaceutical Sciences 15:256 – 264.

Sewalk, C. J., Brewer, G. L. & Hoffman, D. J. (2001) Effects of diquat, an aquatic herbicide, on the development of mallard embryos. Journal of Toxicology and Environmental Health A 62:33–45.

Simons M.J.P., Cohen A.A., Verhulst S. (2012) What does carotenoid-dependent coloration tell? Plasma carotenoid level signals immunocompetence and oxidative stress state in birds - a meta-analysis. PlosOne 7:e43088.

Stahl W., Sies H. (2003) Antioxidant activity of carotenoids. Molecular Aspects of Medicine 24:345–351.

Stahl W., Sies H. (2005) Bioactivity and protective effects of natural carotenoids. Biochimica et Biophysica Acta 1740:101– 107.

Sohal R.S., Orr W.C. (2012). The redox stress hypothesis of aging. Free Radical Biology and Medicine 52:539–555.

Sorci G., Faivre B. (2009) Inflammation and oxidative stress in vertebrate host-parasite systems. Philosophical Transactions of the Royal Society B 364:71-83.

Stirnemann I., Johnston G., Rich B., Robertson J. Kleindorfer S. (2009) Phytohaemagglutinin (PHA) response and bill-hue wavelength increase with carotenoid supplementation in Diamond Firetails (*Stagonopleura guttata*). Emu 109:344–351.

Stradi R. (1998) The Colour of Flight: Carotenoids in Bird Plumage. Solei Gruppo Editoriale Informatico, Milan.

Stradi R., Pini E., Celentano, G. (2001) Carotenoids in bird plumage: the complement of red pigments in the plumage of wild and captive bullfinch (*Pyrrhula pyrrhula*). Comparative Biochemistry and Physiology B 128:529-535.

Surai P.F., Speake B., Wood N., Blount J.D., Bortolotti G., Sparks N.H.C. (2001) Carotenoid discrimination by the avian embryo: a lesson from wild birds. Comparative Biochemistry and Physiology B 128:743–750.

Surai P.F. (2012) 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 Science Journal 68:465–476.

Tyssandier V., Cardinault N., Caris-Veyrat C., Amiot M.J., Grolier P., Bouteloup C., Azais-Braesco V., Borel P. (2002) Vegetable-borne lutein, lycopene, and β-carotene compete for incorporation into chylomicrons, with no adverse effect on the medium-term (3-wk) plasma status of carotenoids in humans. The American Journal of Clinical Nutrition 75:526–34.

Toomey M.B., McGraw K.J. (2010) The effects of dietary carotenoid intake on carotenoid accumulation in the retina of a wild bird, the house finch (*Carpodacus mexicanus*). Archives of Biochemistry and Biophysics 504:161-168.

Toomey M.B., McGraw K.J. (2011) The effects of dietary carotenoid supplementation and retinal carotenoid accumulation on vision-mediated foraging in the house finch. PLosOne 6:e21653 .

Toomey M.B., McGraw K.J. (2012) Mate choice for a male carotenoid-based ornament is linked to female dietary carotenoid intake and accumulation. BMC Evolutionary Biology 12:3.

Vallverdú-Coll, N., Ortiz-Santaliestra, M.E., Mougeot, F., Vidal, D., Mateo, R. (2015). Sublethal Pb exposure produces season-dependent effects on immune response, oxidative balance and investment in carotenoid-based coloration in red-legged partridges. Environmental Science and Technology 49:3839-3850.

van den Berg H. (1999) Carotenoid interactions. Nutrition Reviews 57:1-10.

van Noordwijk A.J., de Jong G. (1986) Acquisition and allocation of resources: their influence on variation in life history tactics. American Naturalist 128:137 – 142.

Velando, A., Drummond, H., Torres, R. (2006) Senescent birds redouble reproductive effort when ill: confirmation of the terminal investment hypothesis. Proceedings of the Royal Society B 273:1443–1448.

Völker O. (1957) Die experimentelle rotfärbung des Gefieders beim Fichtenkreuzschnabel (*Loxia curvirostra*). Journal of Ornithology 98:210-214.

von Schantz T., Bensch S., Grahn M., Hasselquist D., Wittzell H. (1999) Good genes, oxidative stress and condition-dependent sexual signals. Proceedings of the Royal Society B 266:1-12.

Williams T.D. (2005) Mechanisms Underlying the Costs of Egg Production. BioScience. 55:39-48.

Xu J., Sun S., Wei W., Fu J., Qi W., Manchester L.C., Tan D.-X., Reiter, R.J. (2007). Melatonin reduces mortality and oxidatively mediated hepatic and renal damage due to diquat treatment. Journal of Pineal Research 42, 166–171.

Zahavi A. (1975) Mate selection—selection for a handicap. Journal of Theoretical Biology 53:205–214.

Zeman, M., Herichová, I., Navarová, J., Gressnerová, S. & Skrobánek, 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.

Table 1: *Composition of a sample of each different type of food used in the experiment. For the names of the diets see section 2.1.*

Name diet	Lutein	Zeaxanthin	Astaxanthin	Total carotenoids	Retinol	Tocopherol
Control	1.33	0.69	0	1.96	2.5	8.3
LutZea	24.77	9.32	0	34.09	3.5	10.9
ZeaLut	17.64	18.6	0	36.24	10.4	15.1
Ast	5.3	4.8	22.87	32.97	14.4	18.5

Table 2: *Mixed models testing the interaction between carotenoid treatment and time. The reported tests are the best fitted models with an interaction at P < 0.10, or instead, when it is removed at higher P-values by following a backward-step wise procedure (see Methods)*

Dependent variable	*Terms in the model*	*Slope*	*SE*	*F*	*df*	*P*
Eye rings redness	Carotenoid			10.22	3, 167	<0.001
	Sex			3.97	1, 167	0.048
	Time			5.43	2, 237	0.005
	Carotenoid x time			2.65	6, 237	0.017
	Eggs	-0.001	0.0005	5.05	1, 237	0.026
	Total brightness	-0.0001	0.00001	18.05	1, 237	<0.001
	Plasma tocopherol	0.026	0.009	7.98	1, 237	0.005
Bill redness	Carotenoid			6.58	3, 168	<0.001
	Time			8.64	3, 235	<0.001
	Carotenoid x time			1.96	6, 235	0.072
	Eggs	-0.0013	0.0005	5.87	1, 235	0.016
	Total brightness	-0.0002	0.00002	63.68	1,235	<0.001
	Plasma tocopherol	0.0328	0.0108	9.23	1, 235	0.003
Legs redness	Carotenoid			6.03	3, 167	<0.001
	Sex			17.60	1, 167	<0.001
	Time			12.44	2, 227	<0.001
	Sex x time			1.36	2,227	0.258
	Carotenoid x time			0.63	6, 227	0.703
	Eggs	-0.025	0.022	1.28	1, 227	0.259
	Red chip	1.251	0.274	20.85	1, 227	<0.001
	Plasma tocopherol	1.129	0.467	5.84	1, 227	0.016
	Plasma retinol	-1.855	1.147	2.62	1, 227	0.107
Plasma lutein	Carotenoid			105.1	3, 164	<0.001
	Sex			54.22	1, 164	<0.001
	Time			69.61	2, 237	<0.001
	Sex x carotenoid			5.22	3, 164	0.002
	Carotenoid x time			36.81	6, 237	<0.001
	Plasma tocopherol	0.456	0.026	313.86	1, 237	<0.001
	Plasma retinol	0.168	0.0648	6.74	1, 237	0.010
	Eggs	-0.004	0.001	8.57	1, 237	0.004
Plasma zeaxanthin	Carotenoid			309.6	3, 164	<0.001
	Sex			47.35	1, 164	<0.001
	Time			73.54	2, 235	<0.001
	Carotenoid x time			95.93	6, 235	<0.001
	Sex x carotenoid			3.68	3, 164	0.013
	Sex x time			4.40	2, 235	0.013
	Plasma tocopherol	0.379	0.026	216.34	1, 235	<0.001
	Plasma retinol	0.188	0.064	8.72	1, 235	0.004
	Eggs	-0.006	0.001	20.90	1, 235	<0.001
Plasma tocopherol	Carotenoid			2.61	3, 167	0.053
	Sex			2.56	1, 167	0.112
	Time			117.68	2, 236	<0.001
	Carotenoid x time			2.63	6, 236	0.017

	Sex x time			4.89	2, 236	0.008
	Plasma retinol	0.548	0.122	20.25	1, 236	<0.001
	Eggs	-0.009	0.002	14.94	1, 236	<0.001
Plasma retinol	Carotenoid			2.11	3, 168	0.101
	Time			36.06	2, 238	<0.001
	Carotenoid x time			1.01	6, 238	0.421
	Plasma tocopherol	0.085	0.019	20.80	1, 238	<0.001
	Eggs	-0.002	0.001	5.14	1, 238	0.024
Uric acid-albumin-corected PLAOX	Carotenoid			7.19	3, 164	<0.001
	Time			0.38	2, 187	0.686
	Carotenoid x time			2.57	6, 187	0.021
	Uric acid	0.7675	0.0533	214	1, 187	<0.001
	Albumin	-0.4979	0.1117	19.99	1, 187	<0.001
	Plasma retinol	0.2275	0.0984	5.17	1, 187	0.024
Plasma TRG-corrected MDA	Carotenoid			1.05	3, 164	0.372
	Sex			0.29	1, 164	0.593
	Time			14.45	2, 228	<0.001
	Carotenoid x time			0.71	6, 228	0.645
	Sex x carotenoid			1.80	3, 164	0.149
	Sex x time			0.78	2, 228	0.460
	Plasma tocopherol	-0.040	0.045	0.82	1, 228	0.366
	Plasma retinol	-0.088	0.108	0.66	1, 228	0.416
	Plasma triglycerides	0.298	0.031	90.14	1, 228	<0.001
	Eggs	0.001	0.002	0.37	1, 228	0.544
Resistance to oxidative stress in erythrocytes	Carotenoid			1.48	3, 163	0.223
	Sex			1.30	1, 163	0.256
	Time			1.70	2, 203	0.185
	Carotenoid x time			0.69	6, 203	0.657
	Sex x carotenoid			0.59	3, 163	0.619
	Plasma retinol	-20.202	8.811	5.26	1, 203	0.023
	Eggs	-0.339	0.180	3.55	1, 203	0.061
	Lag time	-0.122	0.021	35.90	1,203	<0.001

Table 3. *Mixed models testing how the exposure to oxidative stress (diquat) interacted with the dietary carotenoid treatment at the end of the experiment. The level of each dependent variable in the sampling event precedent to the diquat exposure is included as a covariate for color and blood variables. The models describe the backward step (using the P=0.10 threshold) previous to remove the diquat x CAR interaction (i.e. when it was non-significant; Methods).*

Dependent variable	*Terms in the model*	*Slope*	*SE*	*F*	*df*	*P*
Eye rings redness	Carotenoid			24.93	3, 147	<0.001
	Diquat			0.03	1, 146	0.859
	Sex			0.80	1, 146	0.373
	Carotenoid x Diquat			1.38	3, 146	0.252
	Sex x Diquat			3.21	1, 146	0.075
	Total brightness	-0.00004	0.00002	6.25	1, 147	0.014
	Eye ring redness in day 48	0.291	0.064	20.95	1, 147	<0.001
	Liver vitamin A	0.0004	0.0003	1.33	1, 147	0.251
	Eye ring tocopherol	0.0251	0.012	4.43	1, 146	0.037
	Eggs during diquat experiment	-0.002	0.0008	4.44	1, 146	0.037
Bill redness	Carotenoid			17.19	3, 83.2	<0.001
	Diquat			4.23	1, 76.6	0.043
	Sex			1.46	1, 75.9	0.231
	Carotenoid x Diquat			1.03	3, 74.3	0.382
	Sex x Carotenoid			1.34	3, 69.7	0.269
	Total brightness	-0.0001	0.00003	23.14	1, 143	<0.001
	Bill redness in day 48	0.151	0.056	7.4	1, 134	0.007
	Bill tocopherol	0.048	0.014	11.7	1, 118	<0.001
	Liver vitamin A	0.001	0.0004	5.72	1, 138	0.018
Leg redness	Carotenoid			10.55	3, 136	<0.001
	Diquat			0.23	1, 137	0.631
	Sex			3.98	1, 138	0.048
	Carotenoid x Diquat			0.67	3, 137	0.575
	Sex x Diquat			0.33	1, 137	0.567
	Sex x Carotenoid			1.69	3, 137	0.173
	Red chip	1.018	0.221	21.28	1, 28.8	<0.001
	Leg redness in day 48	0.594	0.065	82.46	1, 137	<0.001
	Leg tocopherol	2.752	0.605	20.73	1, 137	<0.001
	Liver tocopherol	-1.469	0.544	7.28	1, 138	0.008
	Liver vitamin A	-0.024	0.015	2.73	1, 135	0.101
	Total number of eggs	-0.032	0.014	5.10	1, 137	0.026
Total astaxanthin in the eye rings	Carotenoid			51.64	3, 146	<0.001
	Diquat			1.47	1, 151	0.227
	Carrotenoid x Diquat			3.21	3, 147	0.025
	Sex			17.40	1, 148	<0.001
	Sex x Carotenoid			3.21	3, 146	0.025
	Tocopherol in eye ring	0.674	0.060	125.22	1, 149	<0.001

	Total number of eggs	-0.004	0.002	6.54	1, 145	0.012
Total papilioerythrinone in the eye rings	Carotenoid			19.9	3, 88.3	<0.001
	Diquat			0.55	1, 80.1	0.460
	Sex			13.83	1, 79.6	<0.001
	Carotenoid x Diquat			0.33	3, 77.4	0.804
	Sex x Diquat			1.74	1, 81.8	0.190
	Sex x Carotenoid			3.71	3, 79.5	0.015
	Fat retinol	0.032	0.031	1.04	1, 136	0.309
	Plasma tocopherol	0.300	0.182	2.71	1, 140	0.102
	Tocopherol in eye ring	0.850	0.149	32.42	1, 140	<0.001
	Total number of eggs	-0.007	0.004	3.03	1, 83.8	0.086
Tocopherol in the eye rings	Carotenoid			3.64	3, 71.7	0.017
	Diquat			1.22	1, 75.7	0.272
	Carotenoid x Diquat			2.63	3, 73.5	0.056
	Total number of eggs	-0.006	0.002	8.96	1, 71.1	0.004
Total astaxanthin in the bill	Carotenoid			141.3	3, 151	<0.001
	Sex			5.43	1, 155	0.021
	Diquat			4.68	1, 157	0.032
	Carrotenoid x Diquat			2.67	3, 155	0.049
	Plasma tocopherol	1.176	0.061	371.2	1, 158	<0.001
	Total number of eggs	-0.007	0.002	17.86	1, 151	<0.001
Total papilioerythrinone in the bill	Carotenoid			134.5	3, 64.8	<0.001
	Diquat			0.08	1, 68.8	0.774
	Sex			2.66	1, 75.8	0.107
	Carotenoid x Diquat			1.76	3, 66	0.163
	Tocopherol in bill	1.536	0.133	133.35	1, 134	<0.001
	Plasma retinol	-9.373	4.689	4.00	1, 140	0.048
	Total number of eggs	-0.009	0.003	9.17	1, 73.4	0.003
Tocopherol in the bill	Carotenoid			2.94	3, 158	0.035
	Diquat			3.91	1, 162	0.050
	Carotenoid x Diquat			3.09	3, 160	0.029
	Sex			5.6	1, 161	0.019
	Total number of eggs	-0.007	0.002	9.66	1, 159	0.002
Total astaxanthin in the legs	Carotenoid			7.36	3, 92.1	<0.001
	Diquat			0.13	1, 78.8	0.7168
	Sex			2.98	1, 86.9	0.088
	Carotenoid x Diquat			0.07	3, 76	0.974
	Sex x Diquat			0.14	1, 76.6	0.712
	Sex x Carotenoid			0.56	3, 74.3	0.645
	Plasma tocopherol	0.146	0.113	1.66	1, 144	0.199
	Liver vitamin A	0.004	0.002	3.32	1, 133	0.071
	Fat retinol	-0.032	0.018	3.19	1, 130	0.077

	Tocopherol in leg	0.526	0.097	29.25	1, 141	<0.001
	Total number of eggs	0.003	0.002	1.37	1, 87.5	0.244
Total papilioerythrinone in the legs	Carotenoid			4.17	3, 92.9	0.008
	Diquat			0.61	1, 84.9	0.436
	Sex			6.93	1, 91.3	0.010
	Carotenoid x Diquat			0.17	3, 82.1	0.919
	Sex x Diquat			0.24	1, 81.1	0.627
	Sex x Carotenoid			0.16	3, 78.8	0.919
	Tocopherol in leg	0.867	0.147	34.64	1, 143	<0.001
	Liver vitamin A	-0.003	0.004	0.49	1, 144	0.484
Tocopherol in the legs	Carotenoid			4.09	3, 82.2	0.009
	Diquat			2.40	1, 83.8	0.125
	Carotenoid x Diquat			1.21	1, 82.4	0.3126
	Sex			5.98	1.89.5	0.016
	Total number of eggs	-0.004	0.002	4.41	1, 82.1	0.039
Plasma lutein	Carotenoid			151.01	3, 149	<0.001
	Diquat			0.01	1, 149	0.925
	Carotenoid x Diquat			2.84	3, 149	0.040
	Lutein at time 2	0.446	0.0620	51.81	1, 149	<0.001
	Plasma tocopherol	0.479	0.0326	215.52	1, 149	<0.001
	Eggs during diquat experiment	-0.003	0.002	4.42	1, 149	0.037
Plasma zeaxanthin	Carotenoid			321.37	3, 146	<0.001
	Diquat			0.83	1, 146	0.363
	Carotenoid x Diquat			1.57	3, 146	0.200
	Zeaxanthin at time 2	0.307	0.070	19.24	1, 146	<0.001
	Plasma tocopherol	0.572	0.039	219.4	1, 146	<0.001
	Fat tocopherol	-0.037	0.013	8.24	1, 146	0.005
	Liver vitamin A	0.002	0.001	4.79	1, 146	0.030
	Eggs during diquat experiment	-0.004	0.002	4.05	1, 146	0.046
Plasma tocopherol	Carotenoid			5.26	3, 95.1	0.002
	Diquat			2.72	1, 82.3	0.103
	Sex			0.14	1, 86.2	0.710
	Carotenoid x Diquat			0.7	3, 79.3	0.552
	Sex x Diquat			0.70	1, 82.7	0.404
	Tocopherol at time 2	0.199	0.069	8.31	1, 139	0.005
	Liver vitamin A	-0.004	0.002	3.99	1, 134	0.048
	Fat retinol	0.030	0.016	3.34	1, 138	0.070
	Plasma retinol	0.259	0.162	2.57	1, 141	0.111
	Total number of eggs	-0.003	0.002	2.21	1, 84.2	0.141
Plasma retinol	Carotenoid			1.36	3, 80.5	0.262
	Diquat			1.54	1, 80.3	0.218

	Sex			0.51	1, 77.9	0.476
	Carotenoid x Diquat			0.51	3, 77.7	0.678
	Sex x Diquat			0.31	1, 78.7	0.579
	Sex x Carotenoid			0.64	3, 79.5	0.589
	Retinol at time 2	33.86	4.270	62.89	1, 134	<0.001
	Plasma tocopherol	5.209	1.950	7.13	1, 140	0.009
	Eggs during diquat experiment	-0.258	0.095	7.34	1, 73.6	0.008
Liver lutein	Carotenoid			128.63	3, 157	<0.001
	Diquat			0.06	1, 157	0.811
	Carotenoid x Diquat			1.49	3, 157	0.220
	Plasma tocopherol	0.075	0.036	4.45	1, 157	0.037
	Liver tocopherol	0.263	0.028	85.5	1, 157	<0.001
Liver zeaxanthin	Carotenoid			315.42	3, 151	<0.001
	Diquat			0	1, 154	0.971
	Carotenoid x Diquat			3.06	3, 151	0.030
	Plasma tocopherol	0.100	0.046	4.69	1, 151	0.031
	Liver tocopherol	0.341	0.040	74.08	1, 40.3	<0.001
	Plasma retinol	0.003	0.001	3.60	1, 153	0.060
Liver tocopherol	Carotenoid			12.77	3, 161	<0.001
	Diquat			6.47	1, 161	0.012
	Carotenoid x Diquat			2.76	3, 161	0.044
Liver vitamin A	Carotenoid			57.35	3, 152	<0.001
	Diquat			0.04	1, 154	0.834
	Sex			22.3	1, 154	<0.001
	Carotenoid x Diquat			0.47	3, 152	0.707
	Sex x Diquat			0.71	1, 152	0.399
	Plasma tocopherol	-11.032	4.001	7.60	1, 153	0.007
	Liver tocopherol	10.309	3.538	8.49	1, 55.8	0.005
	Total number of eggs	-0.394	0.070	31.26	1, 152	<0.001
Fat lutein	Carotenoid			12.87	3, 147	<0.001
	Diquat			0.06	1, 148	0.808
	Sex			0.13	1, 147	0.716
	Carotenoid x Diquat			0.21	3, 148	0.890
	Sex x Diquat			0.19	1, 147	0.662
	Sex x Carotenoid			1.26	3, 147	0.292
	Fat tocopherol	1.288	0.181	50.69	1, 142	<0.001
	Liver vitamin A	0.022	0.010	4.62	1, 147	0.033
	Plasma retinol	-0.039	0.016	5.77	1, 147	0.018
	Fat retinol	0.241	0.087	7.72	1, 148	0.006
Fat zeaxanthin	Carotenoid			46.74	3, 148	<0.001
	Diquat			0.16	1, 148	0.687
	Sex			0.28	1, 147	0.598
	Carotenoid x Diquat			0.12	3, 148	0.948

	Sex x Diquat			0.88	1, 148	0.349
	Sex x Carotenoid			1.23	3, 148	0.302
	Fat tocopherol	0.911	0.150	37.07	1, 129	<0.001
	Liver vitamin A	0.019	0.010	4.55	1, 148	0.035
	Plasma retinol	-0.023	0.014	3.01	1, 148	0.085
	Fat retinol	0.203	0.073	7.84	1, 148	0.006
Fat tocopherol	Carotenoid			0.57	3, 95.7	0.638
	Diquat			1.30	1, 82.8	0.257
	Sex			0.04	1, 81.4	0.849
	Carotenoid x Diquat			0.15	3, 82	0.931
	Sex x Diquat			0.31	1, 81	0.578
	Sex x Carotenoid			1.71	3, 79.4	0.171
	Liver vitamin A	0.005	0.004	1.11	1, 130	0.295
	Plasma retinol	-0.012	0.007	3.05	1, 138	0.083
	Total number of eggs	-0.005	0.005	0.99	1, 91.4	0.323
Fat retinol	Carotenoid			29.11	3, 149	<0.001
	Diquat			0.18	1, 148	0.671
	Sex			0.14	1, 147	0.706
	Carotenoid x Diquat			0.24	3, 149	0.867
	Sex x Diquat			0.05	1, 149	0.816
	Sex x Carotenoid			1.06	3, 148	0.368
	Plasma tocopherol	0.640	0.535	1.43	1, 149	0.234
	Liver tocopherol	0.136	0.428	0.10	1, 149	0.752
	Fat tocopherol	-0.103	0.165	0.39	1, 64.6	0.537
	Total number of eggs	-0.025	0.010	6.77	1, 148	0.010
UA-ALB-corrected PLAOX	Carotenoid			3.4	3, 79.9	0.022
	Diquat			0.37	1, 71.4	0.543
	Sex			1.61	3, 69.7	0.209
	Carotenoid x Diquat			1.34	1, 69.6	0.269
	Carotenoid x Sex			0.33	3, 62.6	0.805
	Diquat x Sex			0.03	1, 75.9	0.855
	Carotenoid x Diquat x sex			2.85	3, 61.3	0.045
	AOX at time 2	0.331	0.110	9.07	1, 73.1	0.004
	Fat tocopherol	0.054	0.030	3.21	1, 103	0.076
	Uric acid	0.056	0.005	119.03	1, 101	<0.001
	Albumin	-0.009	0.005	3.73	1, 104	0.056
	Liver vitamin A	-0.004	0.002	3.69	1, 87.2	0.058
	Eggs during diquat experiment	-0.011	0.005	4.66	1, 83.9	0.034
Plasma TRG-corrected MDA	Carotenoid			0.29	3, 139	0.836
	Diquat			6.84	1, 139	0.009
	sex			4.8	1, 140	0.030
	Carotenoid x Diquat			0.86	3, 139	0.466
	Diquat x Sex			4.45	1, 140	0.037
	TRG-corrected MDA at time 2	2.419	0.745	10.48	1, 139	0.002
	Triglycerides	0.004	0.001	36.4	1, 140	<0.001
	Eggs during diquat experiment	0.126	0.044	8.28	1, 140	0.005

Liver MDA	Carotenoid			1.27	3, 88	0.289
	Diquat			0.2	1, 77.4	0.659
	Sex			22.76	1, 80.4	<0.001
	Carotenoid x Diquat			1.96	3, 76.7	0.127
	Carotenoid x Sex			1.21	3, 75	0.311
	Diquat x Sex			0.15	1, 75.4	0.699
	Carotenoid x Diquat x sex			4.65	3, 76	0.005
	Liver vitamin A	0.0002	0.0001	7.07	1, 138	0.009
	Plasma tocopherol	0.0003	0.0001	3.73	1, 139	0.055
Heart MDA	Carotenoid			0.09	3, 157	0.963
	Diquat			0.95	1, 157	0.331
	Sex			2.75	1, 157	0.099
	Carotenoid x Diquat			1.79	3, 157	0.151
Erythrocyte resistance to oxidative stress	Carotenoid			0.35	3, 61.1	0.793
	Diquat			5.66	1, 61.1	0.021
	Carotenoid x Diquat			2.27	3, 61.4	0.090
	Lag time	-0.229	0.035	44.06	1, 121	<0.001

Table 4. *Best fitted models obtained when the diquat x CAR interaction is removed at P > 0.10 after a backward stepwise procedure (see Methods). Heart MDA and fat tocopherol did not maintained any term (all* P > *0.10).*

Dependent variable	*Terms in the model*	*Slope*	*SE*	*F*	*df*	*P*
Eye rings redness	Carotenoid			25.55	3, 154	<0.001
	Total brightness	-0.00004	0.00002	7.71	1, 155	0.006
	Eye ring redness in day 48	0.286	0.064	19.99	1, 155	<0.001
	Eye ring tocopherol	0.0230	0.012	6.72	1, 154	0.011
	Eggs during diquat experiment	-0.002	0.001	6.21	1, 155	0.014
Bill redness	Carotenoid			16.22	3, 85.7	<0.001
	Diquat			4.46	1, 77.9	0.038
	Total brightness	-0.0001	0.00003	22.32	1, 150	<0.001
	Bill redness in day 48	0.163	0.054	9.10	1, 141	0.003
	Bill tocopherol	0.045	0.013	11.60	1, 132	<0.001
	Liver vitamin A	0.0008	0.0004	4.26	1, 141	0.041
Leg redness	Carotenoid			10.86	3, 77.2	<0.001
	Sex			2.86	1, 80.3	0.095
	Red chip	1.035	0.217	22.83	1, 27.4	<0.001
	Leg redness in day 48	0.580	0.064	81.19	1, 147	<0.001
	Leg tocopherol	3.022	0.589	26.36	1, 146	<0.001
	Liver tocopherol	-1.780	0.523	11.59	1, 147	<0.001
Total papilioerythrinone in the eye rings	Carotenoid			25.34	3, 153	<0.001
	Sex			15.53	1, 156	<0.001
	Tocopherol in eye ring	0.953	0.140	46.43	1, 158	<0.001
	Eggs (total)	-0.009	0.004	5.43	1, 153	0.021
Total papilioerythrinone in the bill	Carotenoid			131.19	3, 68.3	<0.001
	Sex			2.90	1, 76.3	0.092
	Tocopherol in the bill	1.564	0.129	147.34	1, 142	<0.001
	Plasma retinol	-9.038	4.666	3.75	1, 145	0.055
	Eggs (total)	-0.009	0.003	8.37	1, 77.9	0.005
Total astaxanthin in the legs	Carotenoid			9.40	3, 77.9	<0.001
	Sex			10.56	1, 87	0.002
	Tocopherol in the leg	0.564	0.081	48.28	1, 159	<0.001
Total papilioerythrinone in the legs	Carotenoid			5.24	3, 84.2	0.002

	Sex			7.39	1, 86.3	0.008
	Tocopherol in leg	0.866	0.139	38.79	1, 152	<0.001
Tocopherol in the legs	Carotenoid			3.97	3, 85.7	0.011
	Sex			5.74	1, 89.2	0.019
	Eggs (total)	-0.004	0.002	4.93	1, 85.4	0.029
Plasma zeaxanthin	Carotenoid			322.78	3, 150	<0.001
	Zeaxanthin at time 2	0.310	0.070	19.60	3, 150	<0.001
	Plasma tocopherol	0.561	0.038	217.91	3, 150	<0.001
	Fat tocopherol	-0.038	0.013	8.72	3, 150	0.004
	Liver vitamin A	0.002	0.001	5.35	3, 150	0.022
	Eggs during diquat experiment	-0.004	0.002	4.06	3, 150	0.046
Plasma tocopherol	Carotenoid			5.78	3, 98.2	0.001
	Diquat			4.26	1, 86.9	0.042
	Tocopherol at time 2	0.190	0.066	8.32	1, 144	0.005
	Liver vitamin A	-0.003	0.001	2.89	1, 147	0.091
	Fat retinol	0.034	0.016	4.81	1, 147	0.029
Plasma retinol	Plasma tocopherol	5.608	2.058	7.43	1, 153	0.007
	Eggs during diquat experiment	-0.278	0.112	6.18	1, 78	0.015
Liver lutein	Carotenoid			130.26	3, 161	<0.001
	Plasma tocopherol	0.071	0.035	3.98	1, 161	0.048
	Liver tocopherol	0.263	0.028	87	1, 161	<0.001
Liver vitamin A	Carotenoid			59.87	3, 157	<0.001
	Sex			23.03	1, 159	<0.001
	Plasma tocopherol	-11.396	3.947	8.34	1, 157	0.004
	Liver tocopherol	9.738	3.418	8.12	1, 58	0.006
	Total number of eggs	-0.396	0.069	32.78	1, 157	<0.001
Fat lutein	Carotenoid			13.30	3, 156	<0.001
	Fat tocopherol	1.290	0.175	54.41	1, 153	<0.001
	Liver vitamin A	0.020	0.010	4.42	1, 157	0.037
	Plasma retinol	-0.036	0.016	5.34	1, 156	0.022
	Fat retinol	0.271	0.084	10.44	1, 157	0.002
Fat zeaxanthin	Carotenoid			46.81	3, 163	<0.001

	Fat tocopherol	0.952	0.143	44.45	1, 146	<0.001
	Liver vitamin A	0.018	0.008	5.32	1, 162	0.022
	Fat retinol	0.208	0.069	9.03	1, 163	0.003
Fat retinol	Carotenoid			35.37	3, 167	<0.001
	Total number of eggs	-0.026	0.010	8.62	1, 165	0.004
Plasma TRG-corrected MDA	Diquat			7.04	1, 145	0.009
	sex			5.13	1, 147	0.025
	Diquat x Sex			4.66	1, 145	0.033
	TRG-corrected MDA at time 2	2.366	0.739	10.26	1, 144	0.002
	Triglycerides	0.004	0.001	38.25	1, 146	<0.001
	Eggs during diquat experiment	0.136	0.043	10.17	1, 146	0.002
Resistance to oxidative stress in erythrocytes	Diquat			5.64	1, 67.4	0.020
	Lag time	-0.242	0.034	49.91	1, 128	<0.001