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Testing the shared-pathway hypothesis in the carotenoid-based coloration of red crossbills

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The mechanisms involved in the production of red carotenoid-based ornaments of vertebrates are still poorly understood. These colorations often depend on enzymatic transformations (ketolation) of dietary yellow carotenoids, which could occur in the inner mitochondrial membrane (IMM). Thus, carotenoid ketolation and cell respiration could share biochemical pathways, favoring the evolution of ketocarotenoid-based ornaments as reliable indices of individual quality under sexual selection. Captive male red crossbills (*Loxia curvirostra* Linnaeus) were exposed to redox-active compounds designed to penetrate and act in the IMM: an ubiquinone (mitoQ) or a superoxide dismutase mimetic (mitoTEMPO). MitoQ can act as an antioxidant but also distort the IMM structure, increasing mitochondrial free radical production. MitoQ decreased yellow carotenoids and tocopherol levels in blood, perhaps by being consumed as antioxidants. Contrarily, mitoTEMPO-treated birds rose circulating levels of the second most abundant ketocarotenoid in crossbills (i.e., canthaxanthin). It also increased feather total red ketocarotenoid concentration and redness, but only among those birds exhibiting a redder plumage at the start of the study, that is, supposedly high-quality individuals. The fact that mitoTEMPO effects depended on original plumage color suggests that the red-ketocarotenoid-based ornaments indicate individual quality as mitochondrial function efficiency. The findings would thus support the shared pathway hypothesis.

KEY WORDS: **Bird coloration, mito-targeted antioxidants, mitoQ, mitoTEMPO, sexual signaling, shared-pathway hypothesis.**

How and why animal conspicuous colorations evolve are recurrent questions from Wallace's aposematism concept (Wallace 1877) and Darwin's sexual selection (Darwin 1871). Among showy colors exhibited by vertebrates, those produced by carotenoid pigments (many yellow-to-red ones) have attracted the most attention due to four particularities of these compounds: (1) they cannot be synthesized in the animal organism from other substrates, being obtained with the diet only, (2) they are theoretically scarce in food resources, which means that would be difficult to acquire, (3) they play physiological roles contributing to maintaining homeostasis (i.e., by acting as pro-vitamin A molecules or antioxidants; von Schantz et al. 1999; Britton et al. 2004; Hill and Johnson 2012), and (4) they may serve as a reliable signal of individual quality shaping female choice (Kodric-Brown 1985). These four points have allowed formulating hypotheses explaining the evolution of colored traits as reliable signals of individual quality in mate choice/intrasexual competition contexts (see also Blount et al. 2009 for aposematism). Such

hypotheses establish trade-offs in the allocation of time/energy to searching for carotenoid-rich food versus self-maintenance, and in the allocation of ingested carotenoids to coloration versus homeostasis (see Endler 1980; Kodric-Brown 1985; Lozano 1994; Grether et al. 1999; von Schantz et al. 1999). These are particular cases of the reproduction versus survival trade-off central to explain the evolution of life-history traits (Braendle et al. 2011). Here, an inefficient trade-off solution would induce disproportionally higher costs for low-quality animals, making carotenoid-based colored traits honest signals of individual quality (Grafen 1990; Hasson 1997; see also recently Penn and Számadó 2020).

In the framework of the general resource trade-off hypothesis (RTOH), it was predicted that high carotenoid investment in coloration would take away antioxidant resources needed to fight-off the reactive oxygen species (ROS) generated by cell respiration (von Schantz et al. 1999; Alonso-Alvarez et al. 2004). These resources could be the carotenoids, whose antioxidant function for birds has, however, been questioned (e.g., Simons et al. 2012), or other antioxidants protecting carotenoids from ROS-induced bleaching (Hartley and Kennedy 2004). The resulting oxidative damage would constitute the cost assuring the reliability of carotenoid-based signals.

However, some authors have argued that dietary carotenoids could not be scarce, at least among avian species (McGraw 2006; Hill and Johnson 2012). That argument would deactivate RTOH, removing those costs derived from the investment of resources in coloration (Hadfield and Owens 2006; Svensson and Wong 2011; Koch and Hill 2018; Koch et al. 2019). Trait production would be cost-free. Particularly, Hill (2011) postulated that, instead of resource allocation trade-offs, a close physiological link between cell respiration efficiency and carotenoid metabolism could explain the evolution of many carotenoid-based traits. Accordingly, some carotenoid-based colored signals could directly transmit the bearer's intrinsic quality (also Hill and Johnson 2012). Thus, these traits would act as quality "indices" instead of pure signals (see terminology in Maynard Smith and Harper 2003 or Biernaskie et al. 2014). The idea was formulated in a broad context, giving a general hypothesis for the evolution of sexual signals termed the "shared-pathway hypothesis" (SPH; Hill 2011).

Although carotenoid must ultimately be sourced from the diet, some animals can transform dietary carotenoids to new carotenoids through oxidative metabolism. The SPH was developed from the particular case of ornaments generated by transforming common dietary yellow carotenoids (i.e., yellow xanthophylls) to red (keto)carotenoids (Johnson and Hill 2013). This transformation would be made by oxidoreductase enzymes supposedly linked to the cell respiratory chain (Johnson and Hill 2013). This theoretical framework was generated from studies in house finches (*Haemorhous mexicanus*). In that species, males exhibit yellow to red plumages produced by xanthophylls (such as β-cryptoxanthin) or ketocarotenoids (mostly 3-hydroxyechinenone; "3HOE"), respectively (Inouye et al. 2001; Hill et al. 2002; Johnson and Hill 2013), and females clearly display significant preferences for the most colorful males (Hill 1990). The question was, nonetheless, first addressed by Otto Völker in 1957. Ornithologists at that time tried to understand why red crossbills (*Loxia curvirostra*) change their plumage color from red to yellow when caged (also Weber 1961). Although the lack of suitable carotenoid substrates in food under captivity was not entirely discarded (Hudon 1994 and Hill 1994's discussions), the ornithologists suspected that other factors played a role. Thus, Völker (1957) proposed that captive birds are unable to correctly perform the redox transformations converting yellow xanthophylls to red ketocarotenoids (β-cryptoxanthin to 3-HOE, such as in house finches) due to lack of space for flying. This potential mechanism was virtually overlooked during decades as apparently unrelated to any evolutionary hypothesis. Since there is no known evidence for sexual selection for red coloration in red crossbills, this species constitutes a suitable model for understanding the physiology of carotenoid transformation, which is critical for understanding the basis for sexual selection for these traits.

Interestingly, a gene coding for an oxidoreductase (ketolase) enzyme candidate for yellow to red carotenoid transformations has recently been described in birds (Lopes et al. 2016; Mundy et al. 2016). Moreover, high levels of yellow and, particularly, red carotenoids have been found in the hepatocyte mitochondria of house finches (Ge et al. 2015). High levels of red ketocarotenoids were also found in the inner mitochondrial membrane (IMM) compared to other mitochondrial compartments (Hill et al. 2019). The liver seems to be the main carotenoid transformation site in house finches, red crossbills, and other Carduelinae species (del Val et al. 2009b and cites therein; Hill and Johnson 2012). The accumulation of red carotenoids into the liver mitochondria might, at least, partially support the shared pathway hypothesis as this organelle is responsible for cell respiration (Hill 2011). Particularly, this might support the "inner mitochondrial membrane carotenoid oxidation hypothesis" (IMMCOH) as a specific case of the SPH explaining the evolution of carotenoid-based signals (i.e., Johnson and Hill 2013).

In the IMMCOH, red ketocarotenoids are considered molecularly similar to ubiquinone (Johnson and Hill 2013). Ubiquinone is crucial for cell respiration, transferring electrons across the respiratory chain (Wang and Hekimi, 2016). Thus, transforming yellow to red pigments could share the machinery involved in the ubiquinone biosynthesis pathway and ubiquinone:ubiquinol redox cycling and, accordingly, affect respiratory pathways (Johnson and Hill 2013). An experimental finding in another classical model, the zebra finch (*Taeniopygia guttata;* Vieillot 1817),

has provided additional support to the IMMCOH. Males bear a conspicuous red bill mostly colored by canthaxanthin and astaxanthin ketocarotenoids (McGraw and Toomey 2010) that are enzymatically obtained at the colored tissue (i.e., not produced in the liver and circulated in blood such as made by house finches, and probably crossbills; see Mundy et al. 2016). Cantarero and Alonso-Alvarez (2017) administered a synthetic compound targeted to mitochondria to captive males. The substance (mitoquinone mesylate or mitoQ; Smith et al. 2003) introduces the redox-active aromatic ring of ubiquinone (i.e., benzoquinone) into the IMM (Murphy and Smith 2007, see Fig. S1). Zebra finches treated with mitoQ improved bill redness (Cantarero and Alonso-Alvarez 2017).

To disentangle the mechanism responsible for producing red ketocarotenoid-based signals is currently a challenge that may favor the advance of animal signaling theory (e.g., Hill 2011). The close link between signal expression and mitochondrial function provides an opportunity for testing alternative ideas to the current trade-off paradigm and costly signaling systems (Weaver et al. 2017). Moreover, it provides an opportunity to explore the concept of the index of quality (Maynard Smith and Harper 2003) beyond the limits of traditional examples of traits that depend or are positively correlated to body size (e.g., insect horns, deer antlers, some calling traits; e.g., Reby and Comb 2003; Emlen et al. 2012).

Here, we tested the impact of two different mito-targeted antioxidants on the plumage color and tissue levels of carotenoids and antioxidant vitamins in males from the same species used by Völker (1957) and Weber (1961) in their seminal studies: the red crossbill. As an alternative to mitoQ, mitoTEMPO (see Dikalova et al. 2010) was tested. MitoTEMPO includes a piperidine nitroxide that recycles ubiquinol (reduced ubiquinone) to ubiquinone (Fig. S1; Trnka et al. 2008). This decreases oxidative stress by diminishing mitochondrial superoxide radical levels (Dikalova et al. 2010). The mitoTEMPO molecule is shorter than mitoQ (Fig. S1), thus reducing the possibilities of destabilizing the IMM, leading to higher, not lower, ROS production, something reported in some studies about mitoQ function (i.e., Doughan and Dikalov 2007; Gonzalez et al. 2014). Thus, we could assume that mitoTEMPO should behave differently than mitoQ, probably involving a less ambiguous antioxidant action.

In the framework of the RTOH, we may predict that mito-targeted antioxidants should favor increased circulating values of substrate (yellow) carotenoids as they would not be so much required in fighting-off ROS or not bleached by high ROS levels (Hartley and Kennedy 2004). We should note that yellow carotenoids are able to neutralize ROS (Miller et al. 1996) and more prone to be bleached by free radicals than red carotenoids (Mortensen and Skibsfeld 1997). Increased availability of yellow carotenoids in blood should allow a higher red ketocarotenoid synthesis and, accordingly, increased redness. Alternatively, increased circulating levels of red ketocarotenoids but not yellow xanthophylls would reveal increased transformation rates at the liver mitochondria, supporting SPH and particularly IMMCOH. Moreover, considering the current signaling theory (e.g., Maynard Smith and Harper 2003; Biernaskie et al. 2014), we predict that the intrinsic individual quality should affect the results. We thus divided the sample by plumage redness before the experiment, categorizing high- or low-redness crossbills that should theoretically represent high- or low-quality individuals (e.g., Galván et al. 2015 for a similar approach). Here, if a resource allocation trade-off exists (RTOH), increased availability of resources (yellow carotenoids) in blood due to mito-targeted antioxidant protection (above) would mostly benefit low-quality birds, allowing them to invest in coloration. However, if low-quality birds are intrinsically constrained to produce the signal, as proposed by the SPH, an increase in carotenoid availability would not be translated to higher ketocarotenoid levels in blood and feathers or increased redness.

Material and Methods

EXPERIMENTAL DESIGN

Male adult red crossbills were captured using mist nets during March 2017 at three different locations (i.e., Isaba, Leire, and Lakuaga) at the Pyrenean region (Northern Navarra; Spain). The age was determined at the field by the molt pattern (Jenni and Winkler 1994). Juveniles were excluded from the study. Forty-seven males were finally used. The birds were transferred to facilities also located at the Pyrenean region on the same day of capture (i.e., Instituto de Formación Agroambiental, IFA; Jaca, Huesca, Spain). All the birds were housed in four contiguous aviaries. These were 5.9 × 2.5 × 2.2 m (length, width, height, respectively; i.e., 14.75 m^2 surface, 32.45 m^3 volume each one). Every aviary was partially covered with a roof (4 m^2) to allow protection during rainy days. Food was composed of *Pinus sylvestris* pine nuts (Vilmorin, France) and cones, as well as sunflower and hemp seeds. Food, water, and grit were all provided ad libitum. Pine branches with leaves were added for behavioral enrichment. The experiment started after one month approximately to allow acclimation (mean 33 days, range 30–56 days). The first manipulation (carotenoid supply) started on May 15, 2017. Birds were captured when required by using a shade net that transformed each aviary in a funnel. This minimized capture stress because less than five minutes were needed to capture all the birds.

DIETARY CAROTENOID SUPPLEMENTATION

Red crossbills are specialized conifer seed feeders, but it is unclear what food item (e.g., pine leaves, resin) is the dietary source of the main substrate carotenoid (i.e., β-cryptoxanthin) involved

in producing the red pigment 3HOE (del Val et al. 2009a; del Val et al. 2009b). Pine cones contain relatively low β-cryptoxanthin amount (<0.1 mg/kg in del Val et al. 2009b), and we did not find other food items that pine nuts into the crossbill gizzard during annual ringing campaigns (Daniel Alonso, personal communication). To discard a loss of redness due to β-cryptoxanthin scarcity, the birds received a dietary supplement. It was made by mixing two products: (1) a dry extract of mandarin (*Citrus unshiu*) that contained β-cryptoxanthin at 0.17 mg/g (ref. 0677, Supersmart; Luxembourg) and (2) a synthetic β-carotene provided in corn oil that contained 291 mg/g of β-carotene and 7 mg/g of tocopherol (β-carotene 30% FS, DSM Nutritional Products, Switzerland). These figures were verified by HPLC analyses, such as reported below. The β-carotene supplement was chosen because this compound is more abundant in crossbill natural food than β-cryptoxanthin (del Val et al. 2009b). Moreover, β-carotene can be transformed into retinol, but also enzymatically modified to echinenone by one oxidation and then to canthaxanthin by another oxidation step or, instead, to β-cryptoxanthin by one oxidation and one hydroxylation (McGraw 2006 p. 215).

The β-carotene solution was diluted in peanut oil (SIGMA-ALDRICH ref. P2144) at 1:100 volumes to reduce its very high concentration. The dry mandarin extract was then added to that solution (19.3 g per 100 mL; see also Supporting Information). All the birds received the carotenoid mix by pipetting 150 μL into the mouth every other day from the cited date until June 18 (36-day period).

A group of five red male crossbills (see color categories below) was used to verify that the carotenoid supplement indeed increased circulating levels of substrate carotenoids. Accordingly, they received the same oil amount (150 μL every other day throughout the experiment) but containing peanut oil only and no additional pigment. They were also injected with saline only (see injection treatment below) to allow comparisons with the remaining birds. At least one bird of this small group was present in each aviary. The five birds were, nonetheless, excluded from the analyses testing antioxidant treatment effects.

THE ANTIOXIDANT TREATMENT

The males receiving the carotenoid supplement were assigned to one of the three redox treatments (control, mitoQ, or mitoTEMPO, $n = 14$ per group; $N = 42$). The number of birds older than one year was fully balanced among these groups (five birds per group). All these birds were subcutaneously injected into the back every other day throughout 20 consecutive days. When measured the day before the first injection, no significant difference among treatments, color categories, or its interaction was detected on the tarsus length, body mass, or size-corrected body mass (i.e., by adding tarsus length as a covariate to a normally distributed GENMOD testing body mass; see SI and also Statistical analyses section). Each injection had 130 μL volume of saline plus MitoQ or MitoTEMPO.

In mitoQ, synthetic ubiquinone introduction into the IMM is attained by joining together the antioxidant benzoquinone with a triphenylphosphonium cation (TPP^+) that favors molecule penetration into the IMM and its accumulation in the matrix side (Murphy and Smith 2007). The active benzoquinone is connected to TPP^+ by a linker group formed by a 10-carbon alkyl chain (i.e. decyl-TPP^+ or $dTPP^+$). However, this chain length seems to favor membrane permeability, inhibiting the electron transport chain (ETC) and inducing higher superoxide radical generation (see Reily et al. 2013; Trnka et al. 2015; Gottwald et al. 2018). Interestingly, zebra finches only treated with $dTPP^+$ developed paler bills than controls (Cantarero and Alonso-Alvarez 2017). In mitoTEMPO, in contrast, the active nitroxide is joined to TPP^+ by a shorter linker group than mitoQ, thus inducing a lower distortion of the IMM structure (Fig. S1).

MitoQ (Phosphonium, (10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)decyl)triphenyl-, methanesulfonate (1:1)) was kindly provided by Prof. Michael P. Murphy, whereas mitoTEMPO was purchased from SIGMA-ALDRICH (2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride; ref. SML0737). MitoQ was administered at 1mM (2.27 mg/Kg/day), which is the dose that induced an increase in bill redness in male zebra finches (Cantarero and Alonso-Alvarez 2017). Concerning mitoTEMPO dosage, we first considered a study where mice reduced mitochondrial superoxide production and oxidative damage in muscles and vascular tissue when they received 1.5 mg/Kg/day in saline subcutaneously injected throughout 12 weeks (Vendrov et al. 2015; see also Nazarewicz et al. 2013 for same dosage and effect as an anticancer molecule). We then performed a pilot study involving 10 male zebra finches randomly assigned to five different concentrations (0, 0.334, 0.668, 1.335, and 2.67 mg/kg/day) subcutaneously injected in saline every 2 days for 3 weeks. The highest dose (2.67 mg/kg/day; 3mM) was finally chosen as we did not find a significant correlation between dose and body mass change (%) suggesting any health impairment (Spearman's $r = 0.320$, $P = 0.367$), and no evident toxicity symptoms (behavior changes, fatigue, lack of alertness). Additionally, the change (%) in redness at the upper bill mandible increased with dosage (Spearmans' $r = 0.82$, $P = 0.01$; see also Cantarero and Alonso-Alvarez 2017 for color analysis methods).

All the crossbills were weighed and photographed May 23. The following day a blood sample (150 μL) was taken from the jugular vein, and birds were injected for the first time. This is 10 days after the start of the carotenoid supplementation, which should have allowed birds to circulate enough substrate carotenoids for transformation. Data obtained in these two days (May 23 and 24) provided the initial values of the

experiment. Two days after the first blood sampling, all the feathers in the rump region were plucked to induce a synchronized feather growth. The injection period ended on June 14, i.e., when every bird had received 11 injections (a 3-week exposure, approximately). A second (final) blood sample was taken on June 12 to determine the final levels of plasma carotenoids under antioxidant exposure. Birds were allowed to end the feather regrowth until June 23, when they were again weighed and photographed, and the new rump feathers were carefully removed for quantifying carotenoids. Feather samples were stored at −80°C. Birds were released in the original location when they were again fully feathered (July 10). Three birds died before the second blood sampling without clear signs of illness or body mass loss (one bird per treatment). Another mitoQ-treated bird died just after that second blood sampling. This mortality did not differ among treatments ($\chi^2 = 0.55$, df = 3, $P = 0.76$). The rump feather sample of one mitoQ-individual could not be analyzed due to problems during the laboratory processing. One control bird was unable to produce enough rump feathers for any analyses. Lastly, two initial and two final plasma samples could not be assessed due to hemolysis or lack of volume (one control, one mitoTEMPO, and two mitoQ birds). Therefore, the final sample sizes were 37, 36, and 35 for color, feather and plasma analyses, respectively. No color category × antioxidant treatment combination included less than five birds, the sample being thus balanced.

COLOR MEASUREMENTS

The red crossbill plumage was photographed (Canon EOS 50) by putting the birds always at the same position and fixed distance from the objective (Canon Macro Lens EF 50 mm; see Supporting Information for additional details). The bird was placed in a prone position by pulling the bill and legs. A standard grey card (Kodak, NY, USA) was used as a reference, placed next to the bird's flank on the board's surface, and always in the same position. Digital photographs were standardized and analyzed using the recently developed "SpotEgg" software, an image-processing tool for automatizing analysis of avian coloration that solves the need for linearizing the camera's response to subtle changes in light intensity (Gómez and Liñán-Cembrano 2017). We have previously shown that picture-based measurements are highly correlated with the redness measurement (i.e., red hue) obtained from portable spectrophotometers (Mougeot et al. 2007; Alonso-Alvarez and Galván 2011). SpotEgg, however, allows the user to manually draw any region and provide information about its coloration, shape, or other features (SI in Cantarero and Alonso-Alvarez 2017 for additional details). As opposed to portable spectrophotometers that analyze a reduced spot (usually 1–2 mm), the measure of a sizeable delimited area makes this tool useful for capturing most of the variability among individuals (Gómez and Liñán-Cembrano 2017). Accordingly, for each animal, the average of red, green, and blue components of the rump surface was calculated. We then determined hue (º) values through the Foley and van Dam (1982) algorithm. High values of hue indicated pale traits. Hence, we reversed these hue values (multiplying by -1) to obtain a more intuitive variable, i.e., "redness." To obtain positive values, we rescaled the variable by adding the minimum negative value. Thus, high feather redness values indicate redder traits (e.g., Cantarero et al. 2019). Repeatability (Lessells and Boag 1987) calculated on a set of digital photographs measured twice ($n = 30$) was very high ($R = 0.99$).

INITIAL COLOR VARIABILITY IN OUR SAMPLE OF BIRDS

Large variability in plumage color was present in our sample. Birds showed body feathers ranging from yellow to red, including intermediate phenotypes such as homogeneous orange or individuals with small patches of red to yellow feathers randomly distributed in the body (patchy birds). These male phenotypes are frequent in this species (del Val et al. 2014). To address this in our analyses, we divided the bird sample by the median of the rump redness at the start of the experiment. This should theoretically classify birds in high- and low-quality signalers (Galván et al. 2015 for a similar procedure). This led to two blocks: 20 high-redness and 22 low-redness birds. We aimed to distribute these color categories among treatments equally but also avoiding biases in body mass, tarsus length or body condition (see Supporting Information). Accordingly, among high-redness birds, eight individuals were controls, and mitoTEMPO and mitoQ groups included six individuals each one. Among low-redness birds, six birds were serum-injected controls, and mitoTEMPO and mitoQ groups included eight birds each one (the contingence table reported: $\chi^2 = 0.76$, df = 2, $P = 0.683$; see also Supporting Information).

Although juveniles were excluded from the study (above), an age category (8–12-month-old vs. older birds) was established among the adult birds by following Jenni and Winkler (1994)'s wing feather descriptions. However, these age categories were not well-balanced with plumage color categories among mitoQ-treated birds as only one low-redness bird was older than one year (1 vs. 7; see Table S1). Therefore, color-related effects in mitoQ-treated birds could partly be due to age differences. However, the color category did not show any significant effect among mitoQ birds (see Results). Moreover, we fixed the cited two-level age factor in every statistical model to control for this variability. Nonetheless, models excluding this factor provided similar results.

SAMPLE PROCESSING

Blood was immediately stored in a cold box (1–6°C) and centrifuged within 6 h (10,000 rcf, 5 min, 4°C). Plasma samples were

then separated and stored at –80°C until the analyses. Carotenoid analysis in plasma was performed as described before in García-de Blas et al. 2011; García-de Blas et al. 2013). Feathers in the regrown rump were plucked and stored at –80°C. When analyzed, these feathers were defrosted, and the yellow-to-red part of each feather was cut by a scissor. The total mass of this feather sample was weighed for each bird (mean 8.4 mg, range 4.4–13.6 mg), and this measure used for concentration calculations. Carotenoids in feather samples were extracted following McGraw et al. 2003; see also details in Supporting Information).

CAROTENOID AND VITAMIN QUANTIFICATION

Although mitochondrial activity was not assessed, the level of specific substrate and product (transformed) carotenoid pigments in blood and feathers were assessed to infer the activity of biotransformation enzymes supposedly located at the IMM. This replacement procedure avoided sacrificing wild birds as a biopsy would have been needed to extract the liver mitochondria. Carotenoids and vitamins A and E in the rump feathers and plasma were analyzed by HPLC (Agilent Technologies 1200 series) with DAD and FLD detectors, following Rodríguez-Estival et al. (2010), García-de Blas et al. 2011; García-de Blas et al. 2013) and Alonso-Alvarez et al. (2018)'s protocols with some modifications (more details in Supporting Information). Standards of lutein, zeaxanthin, canthaxanthin, astaxanthin, 3HOE, β-cryptoxanthin, and β-carotene were purchased from CaroteNature (Lupsingen, Switzerland). Retinyl acetate (used as an internal standard), retinol and α-tocopherol standards, were provided by Sigma–Aldrich. Concentrations were determined from standard calibration curves and linear calibration adjustments. Saponification with KOH did not provide an additional pigment amount (see saponification method in García-de Blas et al. 2011). Carotenoid and vitamin concentrations in plasma or feathers were expressed as nmol/mL and nmol/g for plasma and feathers, respectively (see also Tables S2 and S3).

STATISTICAL ANALYSES

First, the difference in circulating carotenoid levels between birds fed or not with the carotenoid supplement was tested by non-parametric Fisher exact tests, Pearsons' χ^2, or Mann-Whitney U tests. This procedure avoided the problem of unbalanced sample sizes (only five birds used as controls here), lack of normality or heteroscedasticity. We should note that the concentration of red ketocarotenoids in plasma showed a high frequency of zero-values, ranging from 36.5% in plasma 3-HOE to 70.7 % in plasma astaxanthin.

Subsequently, generalized linear models (GLMs) were used to test the antioxidant treatment effect and its interaction with the (two) color categories ($N = 35$–37, see above). The age category was also fixed (see above). The GENMOD procedure in SAS 9.4 software was used. This allowed specifying the distribution type. Sample sizes and variances were here well balanced among groups. However, zero inflation prevented to use a normal distribution in most ketocarotenoids. In these variables, the adjustment to zero-inflated Poisson or, instead, negative binomial distributions were tested (e.g., Ridout et al. 1998). Only the zero-inflated negative binomial (ZINB in SAS) distribution met the no over-dispersion criterion tested by Scaled Pearson's χ^2, being accordingly used. The dependent variables in ZINB models were not transformed (raw data used). In contrast, plasma yellow xanthophylls, plasma vitamins (α- and γ-tocopherol fractions were summed to obtain total tocopherol) and rump redness were all normally distributed, being tested by GENMOD with normal distribution. Nonetheless, other variables previously required transformations to attain normality. Thus, the sum of the five unknown plasma xanthophylls (UX-xanthophylls; see below) and the sum of all red ketocarotenoids in feathers were log-transformed. Feather levels of β-cryptoxanthin and an unknown derivative of β-cryptoxanthin ("UD-β-cryptoxanthin") required a rank-based inverse normal transformation (Blom 1958), whereas feather canthaxanthin levels were square root-transformed. Feather astaxanthin was only found in two birds and was not tested. Finally, the body mass change (%) from the start of the antioxidant treatment to the second blood sampling or, instead, to the date of color measurement, were normally distributed and tested by GENMOD for coherence.

The use of the GENMOD procedure prevented to include the aviary as a random factor. However, the three treatment groups ($\chi^2_6 = 2.56$, $P = 0.861$), the two color categories ($\chi^2_3 = 0.46$, $P = 0.927$), or the six groups resulting from its interaction ($\chi^2_{15} = 0.46$, $P = 0.902$) were well-balanced among the aviaries.

With regard to covariates, the carotenoid level at the first blood sampling was tested in GENMOD models analyzing carotenoid concentration variability. In the case of the rump color, the initial redness (before plucking) was not added as a covariate because the color category captured its variability. Instead, saturation (purity) and brightness (lightness) were included as covariates to assess color variability fully independent of these parameters (Fitze and Richner 2002). In any event, the interaction between color category and treatment detected in the model (below) remains significant when these two terms are removed (i.e. $P = 0.025$). Initial body mass was excluded as a covariate in models testing body mass change. Similar results were obtained when testing final body mass controlled for an initial-weight covariate, but we considered that body mass change provides more interpretable information. Body mass change models included tarsus length as a covariate to correct for body size variability.

Satterthwaite-corrected DFs and least-square means ± SEs are reported. *Post hoc* comparisons were made by LSD tests.

Cohen's *d* effect sizes for pairwise comparisons were reported when significant (see also Table S4 in SI). An effect size larger than 1 means that the difference between two means is larger than one standard deviation and is conventionally considered a large effect (Cohen 1992).

ETHICS

This study was approved by the Bioethical Committees of CSIC (Ref. 404/2016) and the local government (Junta de Comunidades de Castilla-La Mancha; Ref. 486728).

Results

DESCRIPTION OF CAROTENOIDS AND VITAMINS IN BLOOD AND FEATHERS

In plasma, β-cryptoxanthin, lutein, astaxanthin (only at the second sampling), canthaxanthin, 3HOE and echinenone were detected (see Table S2 for concentrations). Five unidentified carotenoids were also found. Maximum wavelengths and retention times of these unidentified compounds (i.e., Table S3) suggested that they were closer to yellow xanthophylls such as lutein or β-cryptoxanthin. Retinol and α- and γ-tocopherol were also detected. Finally, we detected β cryptoxanthin, an unidentified derivative (probably an isomer) of β-cryptoxanthin ("UD-β-cryptoxanthin"; see Supporting Information), lutein, astaxanthin, canthaxanthin, 3HOE and echinenone in rump feathers. Neither retinol or tocopherol was found in feathers.

SUPPLEMENTARY CAROTENOID EFFECTS

In terms of plasma values, β-cryptoxanthin differed between both groups at both the first and second sampling events ($U = 0.01$, $P = 0.001$ and $U = 8.00$ $P = 0.001$, respectively; other pigments $P > 0.12$). Carotenoid-supplied birds showed β-cryptoxanthin values several times higher than controls at both the first (×12 times: median, range: 0.34, 0.097–0.784 vs. 0.028, 0–0.075 nmol/mL, respectively) and second sampling (×6 times: 0.47, 0–1.063 vs. 0.075, 0–0.312 nmol/mL). No other plasma carotenoid differed (all $P > 0.123$). Additionally, retinol ($U = 41.0$, $P = 0.045$) and total tocopherol ($U = 33.0$, $P = 0.018$) differed at the second blood sampling, with those birds receiving the supplement showing higher and lower levels than controls, respectively (retinol: 5.74, range 3.13–9.98 vs. 4.43, range 3.98–6.12 nmol/mL; tocopherol: 180.48, range 0–383.6 vs. 241.97, range 179.2–330.5 μM).

In terms of feather composition, no control bird for the dietary supplement (those only receiving peanut oil) deposited β-cryptoxanthin or echinenone in the rump (both absence/occurrence Pearsons' χ^2-tests showed $P < 0.01$). The other pigments were found in both groups. When variability in concentrations was assessed, the effect on β-cryptoxanthin and echinenone was also significant (Mann-Whitney $U = 10$, $P = 0.001$ and $U = 30$, $P = 0.013$, respectively). Among other feather pigments, only canthaxanthin showed a trend to significance ($U = 44$, $P = 0.069$), with supplement birds showing higher values (mean, range: 4.82, 0–17 vs. 2.12, 0–6 nmol/g, respectively). Finally, the redness of the moulted rump differed as expected ($U = 21$, $P = 0.005$; supplemented birds: 2.36, 0.75-5.56; controls: 0.87, 0–1.78).

No bird attained an intense red rump, even when supplied with high levels of carotenoids in food (see Supporting Information). The color of the molted rump declined 35.9% in the whole of the sample, ranging from yellow to pale orange. The percentage of red pigments in feathers only reached 22% approximately of the total amount of carotenoids (Table S2).

MITO-TARGETED ANTIOXIDANT EFFECTS ON BODY MASS

The birds involved in the antioxidant experiment gained body mass from captivity to the first injection (mean ± SE: 3.32 ± 0.82%). From the latter date, the treatment affected body mass change to the second (last) blood sampling ($\chi^2 = 11.62$, df = 2, $P = 0.003$; tarsus length covariate: $\chi^2 = 4.76$, df = 1, $P = 0.029$; age: $\chi^2 = 11.25$, df = 1, P = 0.001). MitoQ-birds gained less mass (even losing mass) compared to controls and mitoTEMPO-birds (both $P < 0.005$; mitoQ: -0.61 % ± 0.85, control: 2.79 % ± 0.83; mitoTEMPO: 3.01 % ± 0.82; control vs. mitoTEMPO: $P = 0.85$). Younger birds gained mass compared to older ones (3.47 % ± 0.64 vs 0.01 % ± 0.78), whereas the color category and its interaction with treatment showed *P-values* > 0.60. When the change was tested to the color measurement date, the treatment was again significant ($\chi^2 = 11.19$, $df = 2$, $P = 0.004$; tarsus length: $\chi^2 = 8.90$, $df = 1$, $P = 0.003$; age: $\chi^2 = 7.85$, $df = 1$, $P = 0.005$). Here, birds in both treatments gained less mass than controls (both $P < 0.020$; control: 5.22 % ± 0.77; mitoQ: 1.63 % ± 0.85, mitoTEMPO; 2.62 % ± 0.76; mitoQ vs. mitoTEMPO: $P = 0.38$). Younger birds gained more mass than older ones (4.52 % ± 0.59 vs 1.79 % ± 0.75), whereas the color category and its interaction with treatment were again non-significant (both *P*-values > 0.22).

MITO-TARGETED ANTIOXIDANT EFFECTS ON BLOOD COMPOSITION

In the case of those yellow xanthophylls potentially used as the substrate for ketocarotenoid production, β-cryptoxanthin levels were the lowest in mitoQ treated birds compared to mitoTEMPO and control birds (Fig. 1A; Table 1). Similar results were found for lutein, UX and total tocopherol (Figs. 1B-D), although the latter also showed a trend to significance in the comparison between mitoTEMPO and control birds (i.e., $P = 0.090$).

Table 1. Generalized linear models testing the impact of mito-targeted antioxidant treatments and their interaction with color category on the level of circulating pigments and vitamins of captive male crossbills.

Dependent variable (Distribution)	Estimate	SE	*df*	χ^2	*P*
β-Cryptoxanthin (Normal)					
Initial value covariate	0.577	0.206	1	8.03	0.005
Age category			1	2.38	0.123
Antioxidant treatment			2	32.50	<0.001
Color category			1	2.04	0.153
Interaction			2	0.48	0.787
Lutein (Normal)					
Initial value covariate	0.545	0.100	1	32.25	<0.001
Age category			1	3.25	0.071
Antioxidant treatment			2	8.89	0.012
Color category			1	0.00	0.960
Interaction			2	0.36	0.837
UD xanthophylls (Normal)					
Initial value covariate	0.6034	0.1065	1	32.10	<0.001
Age category			1	2.05	0.152
Antioxidant treatment			2	6.42	0.040
Color category			1	0.10	0.753
Interaction			2	0.31	0.858
Total tocopherol (Normal)					
Initial value covariate	0.6960	0.1549	1	20.18	<0.001
Age			1	0.41	0.523
Antioxidant treatment			2	20.77	<0.001
Color category			1	1.34	0.247
Interaction			2	1.08	0.584
Canthaxanthin (ZINB)					
Initial value covariate	0.0002	0.0001	1	5.81	0.016
Age category			1	1.18	0.278
Antioxidant treatment			2	6.74	0.034
Control	0.245	0.222			
MitoQ	0.293	0.169			
Color category			1	2.54	0.111
Interaction			2	1.71	0.424
Zero inflation model					
Initial value covariate	−0.003	0.001	1	11.57	0.001
Age category	2.129	1.192	1	3.93	0.048
Antioxidant treatment			2	1.64	0.441
Color category			1	2.82	0.093
Interaction			2	2.76	0.251
3-HOE (ZINB)					
Initial value covariate	0.002	0.001	1	7.79	0.005
Age category			1	0.85	0.356
Antioxidant treatment			2	13.67	0.001
Color category			1	2.19	0.139
Interaction			2	7.62	0.022
Zero inflation model					
Initial value covariate	−0.012	0.006	1	2.94	0.086
Age category			2	2.86	0.091
Antioxidant treatment			1	4.69	0.096

(*Continued*)

Table 1. Continued.

Dependent variable (Distribution)	Estimate	SE	*df*	χ^2	*P*
Color category			1	0.01	0.999
Interaction			2	0.28	0.871

Note: Likelihood Ratio χ^2 tests and *P*-values are shown. Type of distribution, normalization procedures if needed, slopes for covariates and log odds for significant terms in ZINB models are also reported. The zero-inflated negative binomial (ZINB) model shows separated outputs for counting and zero-inflated parts of the models. Bold used to mark significant *P*-values involving the treatment factor.

Regarding red ketocarotenoids, mitoTEMPO-treated crossbills showed higher plasma canthaxanthin levels than other groups (Table 1, Fig. 1E). Mitotempo-treated birds showed 28 and 34% higher plasma values than controls and mitoQ-treated birds, respectively (i.e., odds ratios: 1.28 and 1.34, respectively). In the zero-inflation model (Table 1), high initial canthaxanthin levels led to lower probabilities of attaining a zero value, and older birds had more probabilities of reporting a zero value than youngers (older birds were 8.4 times a likely to have no plasma canthaxanthin than younger birds).

The model testing 3HOE detected a significant interaction between color category and treatment (Table 1). Low-redness mitoTEMPO birds showed significantly higher levels than high-redness birds in the same treatment (Fig. 2, Cohen's $d = 1.29$). Low-redness mitoTEMPO birds also showed significantly higher 3HOE values than most of the other groups (d range: 1.73-2.07; effect sizes in Table S4). The other two red pigments in plasma (i.e., astaxanthin and echinenone) and retinol did not show significant effects being not shown (all P-values > 0.10). Note that these two pigments were, anyway, scarce (Table S2).

MITO-TARGETED ANTIOXIDANT EFFECTS ON FEATHER PIGMENTS

In rump feathers, the treatment factor exerted a significant effect on β-cryptoxanthin and echinenone concentrations (Table 2), with mitoQ-birds showing lower levels than the other groups (Figs. 3AB). In the echinenone model (ZINB), comparisons of mitoQ-treated birds with controls or mitoTEMPO-treated birds reported 1.70 and 1.78 odds ratios, respectively.

No significant treatment effect or interaction was detected in feather lutein and canthaxanthin variability (all P's > 0.12), the models being not reported. The same was found regarding feather 3HOE levels (all P's > 0.40), although high-redness birds tended to show higher 3HOE concentrations than low-redness ones (27.78 ± 6.29 and 17.46 ± 3.39 nmol/g, respectively: $\chi^2 = 2.71$, $P = 0.099$).

However, when the total level of red pigments in feathers was tested, the interaction did not reach significance (Table 2). Nonetheless, high-redness birds treated with mitoTEMPO reported significantly higher values than high-redness controls (*Cohen's* $d = 1.31$) and low-redness mitoQ birds ($d = 1.09$; Fig. 4A). Moreover, if an alternative model excluding mitoQ-birds is tested, the interaction tended to significance ($P = 0.060$). On the other hand, a similar model but testing the sum of yellow carotenoids did not detect any significant treatment or interaction effect (all $P > 0.20$).

MITO-TARGETED ANTIOXIDANT EFFECTS ON RUMP REDNESS

The rump redness showed an interaction between the color category and treatment (Table 2 and Fig. 4B). Agreeing with feather red pigments (Fig. 4A), the rump of high-redness mitoTEMPO-birds was significantly redder than that of high-redness controls (Cohen's $d = 1.82$; Fig. 4B). High-redness mitoTEMPO-birds also showed significantly redder rumps than low-redness mito TEMPO-birds or mitoQ-birds, and a trend to significantly redder rumps than low-redness controls ($P = 0.088$; Fig. 4B; Table S4).

Discussion

Our results support the IMMCOH assumption stating that the mitochondrial membrane is the cell site where yellow carotenoids are biotransformed. We here assume the specific action of our mito-targeted antioxidants on the IMM (Murphy and Smith 2007; Dikalova et al. 2010; Zielonka et al. 2017). MitoTEMPO was able to increase the circulating levels of main red ketocarotenoids (i.e., canthaxanthin and also 3HOE among low-redness birds) that could only be obtained by transforming yellow xanthophylls. MitoTEMPO also increased total ketocarotenoid levels in feathers and rump redness, most effects being influenced by individual quality (i.e. initial color). The latter would agree with the SPH as only high-redness (high-quality) individuals were able to produce the ornament at the highest intensity. However, the mitoQ-induced effects might, at least partially, support the presence of a resource allocation trade-off influencing ornament expression (RTOH).

Independently of color categories, our birds gained body mass, probably due to reduced activity and ad libitum food.

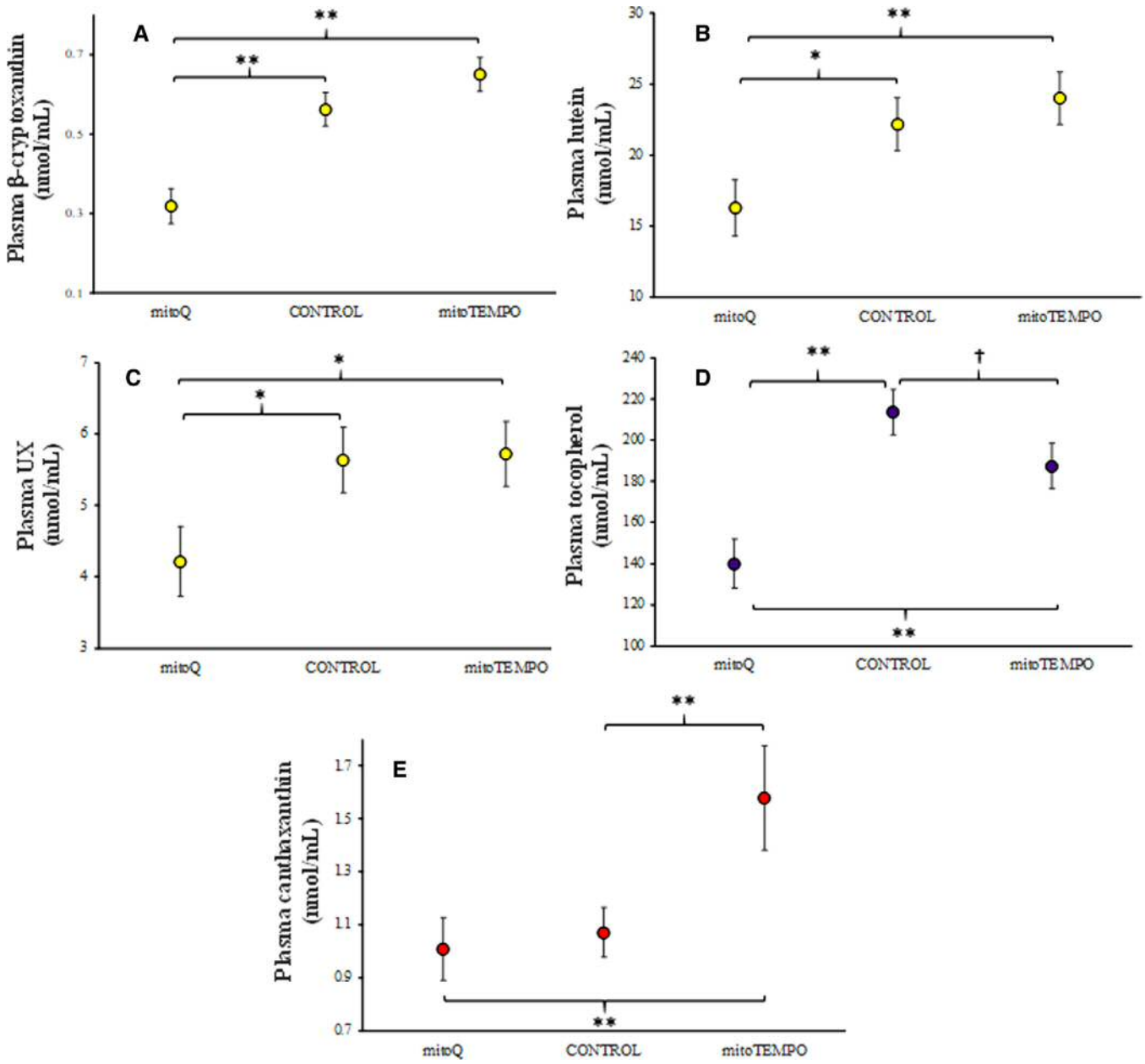

Figure 1. Circulating levels of carotenoids and tocopherol in Eurasian crossbills after treatment with mito-targeted antioxidants. UX: unidentified xanthophylls. Least square means ± SEs from models. LSD pairwise tests: $^{**}P < 0.01$, $^{*}P < 0.05$. Cohen's *d* effect sizes in Table S4.

However, those treated with mito-targeted antioxidants did not gain so much weight as controls. This was first found in mitoQ-treated crossbills. The same mitoQ-induced reduction was detected in male zebra finches (Cantarero and Alonso-Alvarez 2017). Nevertheless, zebra finches only treated with the $dTPP^{+}$ did not report this, suggesting that reduced mass gain is due to changes in redox activity (Cantarero and Alonso-Alvarez, unpublished results). We may infer that ubiquinone increased cell respiration rates, leading to consuming energy stores. Thus, ubiquinone is known to reduce fatness in mammalian models (Allen and Vickers 2014 and references therein), and mitoQ and mitoTEMPO injections have also shown to reduce body mass in obese rodents (Jeong et al. 2016; Fink et al. 2017; Gutiérrez-Tenorio et al. 2017).

MitoQ-birds also showed lower plasma levels of tocopherol and yellow xanthophylls (Fig. 1). The effect could be due to lower food intake. However, when body mass change was added as a covariate, it always reported *P*-values > 0.25. Tocopherol variability was significantly associated with body mass change, but inversely, decreasing with mass gain (body mass gain covariate:

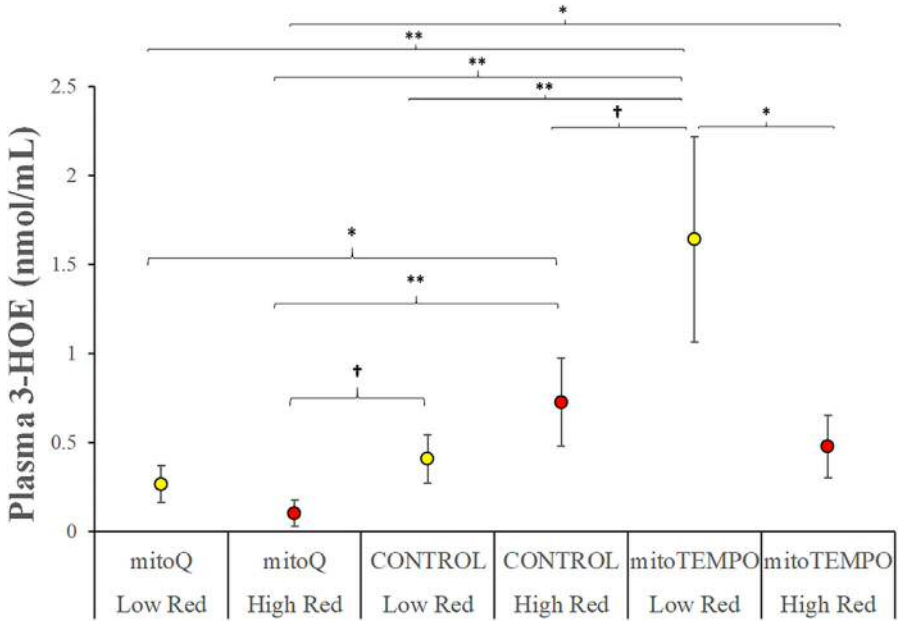

Figure 2. **Variability in circulating levels of the red ketocarotenoid 3HOE in red crossbills after treatment with mito-targeted antioxidants. High- or low-redness groups identify those birds over or below the median value of rump redness at the start of the experiment. Least square means ± SEs from models. LSD pairwise tests: **: $P < 0.01$, *: $P > 0.05$; †: $P < 0.10$. Cohen's *d* effect sizes in Table S4.**

$\chi^2 = 12.48$, $P = 0.001$; slope ± SE: –6.37 ± 1.80; the treatment effect remained significant at $P < 0.001$). We may, alternatively, propose that mitoQ reduced carotenoid and tocopherol intestinal absorption. However, literature in mammals, contrarily, suggests that mito-targeted antioxidants protect the functionality of intestinal cells (e.g., Wang et al. 2014; Hu et al. 2018). As a third option, mitoQ may have increased ROS production and oxidative stress due to its long alkyl linker group (Fig. S1; Maroz et al. 2009; Reily et al. 2013; Trnka et al. 2015; Yasui et al. 2017; Gottwald et al. 2018). This may have promoted the consumption of tocopherol and xanthophylls to be used as radical scavengers (Britton et al. 2004; Panda and Cherian 2014). Unfortunately, no measure of oxidative damage was tested, and hence, no firm conclusion can be made here.

In any event, we can infer that the reduction in circulating levels of yellow carotenoids (substrate to ketocarotenoid synthesis) might constrain coloration, implying that carotenoids could be a limiting resource at the organism level. Hence, crossbills' red coloration might indicate the individual capacity to maintain enough high yellow carotenoid values in blood, supporting the evolution of these traits as reliable signals due to a resource allocation trade-off (i.e., the RTOH; e.g., von Schantz et al. 1999; Alonso-Alvarez et al. 2004). However, although mitoQ-treated crossbills reduced the circulating levels of one ketocarotenoid (i.e., echinenone; Fig. 3), feather ketocarotenoid levels and rump redness did not differ from controls. The decline in substrate carotenoid levels was thus not translated to signal expression. Therefore, compared to mitoTEMPO-induced effects supporting the SPH, mitoQ effects would provide weaker support for the RTOH.

Moreover, the negative (or non-significant) effects of mitoQ on our crossbills contrast with the positive effect of this antioxidant on the bill redness of male zebra finches (i.e., Cantarero and Alonso-Alvarez 2017). This highlights the complexity and diversity of carotenoid-based signaling mechanisms (McGraw 2006). Zebra finches and red crossbills differ in the type of carotenoid-based ornament (bill vs. plumage, respectively) and the carotenoid biotransformation tissues (bill vs. liver; del Val et al. 2009a, b; Mundy et al. 2016). We should consider that the liver is a vital organ devoted to detoxification and other critical functions. Our mitoQ treatment, even when administered at a similar dosage in both species, could have imposed an extra-cost leading to higher ROS, reducing yellow xanthophyll levels and preventing to detect a positive effect on bird color.

In contrast to mitoQ, mitoTEMPO raised the plasma levels of the two most abundant ketocarotenoids of our red crossbills, that is, 3HOE and canthaxanthin (Table S2). MitoTEMPO-birds should, thus, have been able to increase the carotenoid transformation rate, probably at the mitochondria (Johnson and Hill 2013). However, the transformation pathway of the two carotenoids differs. Whereas 3HOE values depend on β-cryptoxanthin availability, canthaxanthin should mostly result from β-carotene oxidation (Fig. S2). Surprisingly, we did not detect β-carotene in the blood, even among our β-carotene

Table 2. Generalized linear models testing the impact of mito-targeted antioxidant treatments and color category on the values of feather carotenoids and rump coloration of captive male Eurasian crossbills.

Dependent variable (Distribution)	Estimate	SE	*df*	χ^2	*P*
Std-β-cryptoxanthin (Normal)					
Age category			1	0.54	0.464
Antioxidant treatment			2	9.53	0.009
Color category			1	1.53	0.216
Interaction			2	1.99	0.370
Echinenone (ZINB)					
Age category			1	3.53	0.060
Antioxidant treatment			2	12.72	0.002
Control	0.531	0.222			
MitoQ	0.579	0.234			
Color category			1	3.13	0.077
Interaction			2	0.26	0.879
Zero inflation model					
Age category			1	0.36	0.551
Antioxidant treatment			2	1.21	0.546
Color category			1	0.40	0.525
Interaction			2	1.38	0.501
Log-Total red ketocarotenoids (Normal)					
Age category			1	2.00	0.158
Antioxidant treatment			2	3.13	0.209
Color category			1	0.06	0.807
Interaction			2	3.73	*0.155*[a]
Rump redness (Normal)					
Age category			1	0.01	0.974
Saturation	6.438	4.875	1	1.74	0.187
Brightness	−6.145	3.264	1	3.55	0.060
Antioxidant treatment			2	3.85	0.146
Color category			1	1.47	0.225
Interaction			2	7.93	0.019

Note: Likelihood Ratio χ^2 tests and *P*-values are shown. Type of distribution, normalization procedures if needed, slopes for covariates and log odds for significant terms in ZINB models are also reported. The zero-inflated negative binomial (ZINB) model separately shows outputs for counting and zero-inflated parts of the models. Bold used to mark significant *P*-values involving the treatment factor. [a]This non-significant interaction is reported to explore pairwise comparisons (Figure 4A).

supplemented birds. The lack of circulating β-carotene has been reported in several avian species, including passerines fed with the pigment (reviewed in McGraw 2006). Interestingly, in American flamingos (*Phoenicopterus ruber*), those birds fed with β-carotene did not show circulating β-carotene but increased blood canthaxanthin levels (Fox et al. 1969). We may, therefore, suggest that mitoTEMPO favored β-carotene transformation at the intestine wall. This should imply the presence of the CYP2J19 monooxygenase (Lopes et al. 2016; Mundy et al. 2016) or some specific β-carotene ketolase at the enterocytes. However, such a possibility has never been reported for any species as far as we know.

In any case, the most abundant red carotenoid in crossbill feathers is 3HOE (17 vs. 5 μM for 3HOE and canthaxanthin, respectively; see Supporting Information and del Val et al. 2009a). Its primary substrate (i.e., β-cryptoxanthin) was well represented in blood and, hence, carotenoid transformation at the liver can, in this case, be defended. Here, low-redness birds treated with mitoTEMPO showed the highest mean plasma values (Fig. 2). At first glance, this could support the prediction (Introduction) that reduced ROS generation could save antioxidants allowing circulating higher carotenoid levels, particularly among low-quality birds. However, yellow xanthophyll values in blood did not increase among mitoTEMPO birds, and neither depending on bird coloration. Yellow xanthophylls like lutein or β-cryptoxanthin are theoretically more able to neutralize free radicals (Miller et al. 1996) and more prone to be degraded when exposed to ROS (Mortensen and Skibsfeld 1997)

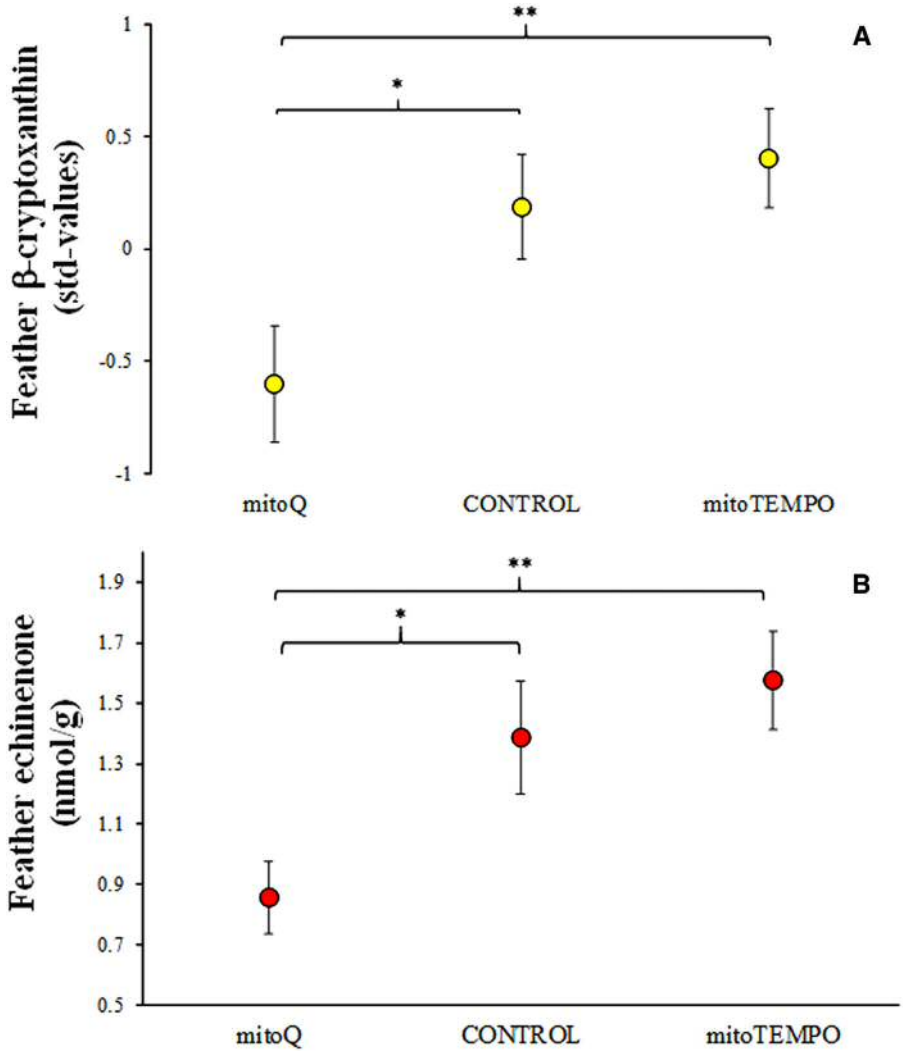

Figure 3. **Variability in feather carotenoid concentration in red crossbills after treatment with mito-targeted antioxidants. Least square means ± SEs from models. LSD pairwise tests: **$P < 0.01$, *$P > 0.05$. Cohen's *d* effect sizes in Table S4.**

compared to red ketocarotenoids. Moreover, only high-redness birds were able to increase feather ketocarotenoid levels and color expression when exposed to mitoTEMPO, which supports that the trait indicates individual quality via mitochondrial metabolism (SPH).

The intriguing finding that high-redness-quality birds treated with mitoTEMPO did not raise plasma 3HOE levels, such as low-redness individuals, should be interpreted with caution. In high-redness birds, mitoTEMPO might have improved the capacity of follicles to capture 3HOE from the bloodstream. For instance, it might have favored the formation of carotenoid-protein complexes in follicle cell membranes, allowing carotenoid uptake (e.g., Reszczynska et al. 2015). The complexes can be composed of antioxidant proteins such as glutathione S-transferase, which has been linked to carotenoid deposition in tissues (Reszczynska et al. 2015; Toews et al. 2017). Such a possibility implies that red ketocarotenoid-based signaling might not only depend on trans-formation rates, and hence, mitochondrial efficiency, as proposed by the SPH. However, mitoTEMPO acts on the IMM (above), not on cell membranes. Therefore, the hypothetical mechanism should require some indirect pathway to influence carotenoid uptake.

Alternatively, we propose a time-based approach. The mito-targeted antioxidant might have induced a slower increase in pigment conversion rate among low-quality paler birds. The animals were blood sampled when virtually all the individuals had ended follicle development (see Supporting Information). Thus, higher plasma 3HOE levels in low-redness mitoTEMPO-treated birds might mean that 3HOE did not arrive in time. The pigment could not be allocated to follicles, being accumulated in the blood. High-redness birds of the same treatment would, contrarily, have depleted the circulating 3HOE pool by previously allocating the pigment to feathers, thus reporting similar levels than controls. If this is correct, higher plasma 3HOE levels in

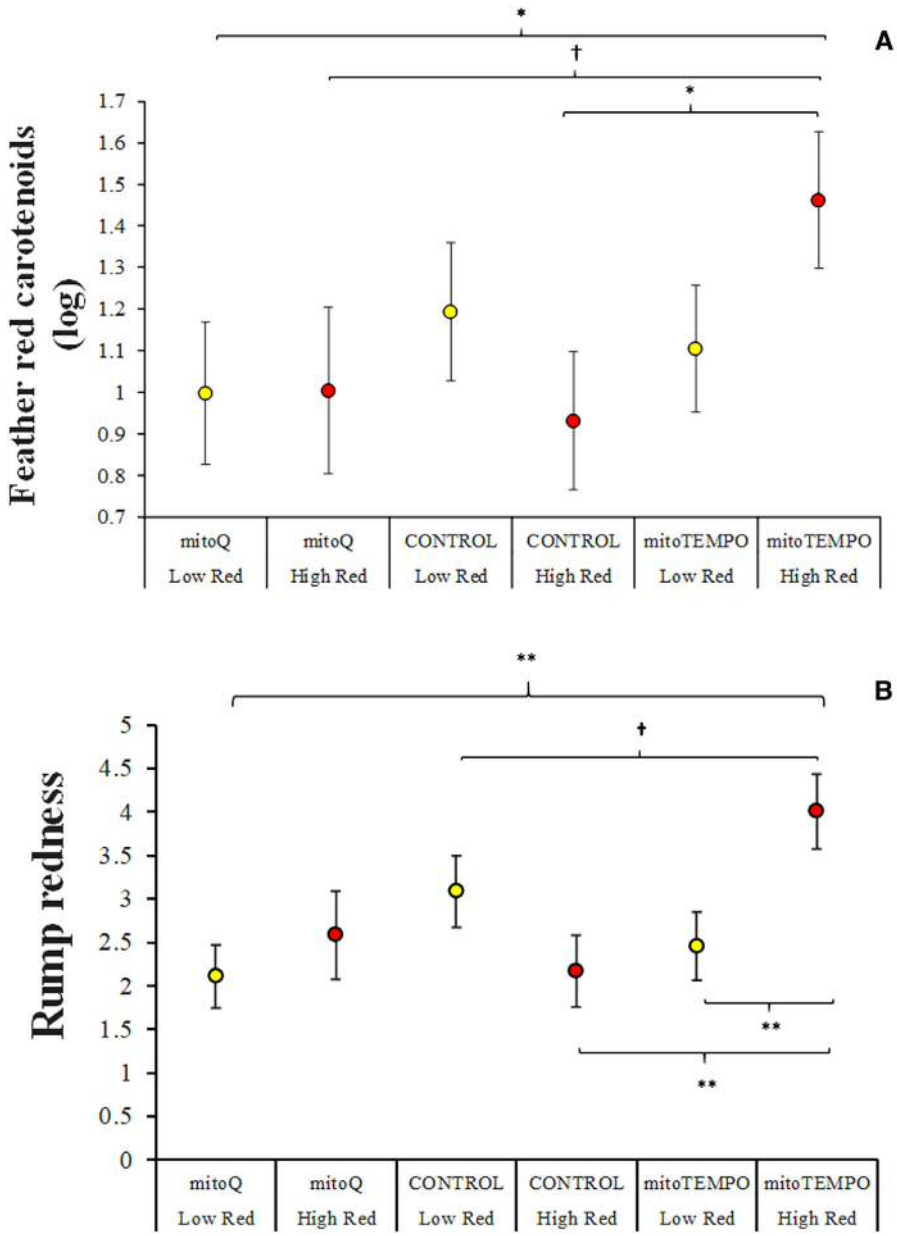

Figure 4. **Variability in the total level of red ketocarotenoids in rump feathers (A) as well as rump redness variability (B) in Eurasian crossbills after treatment with two different mito-targeted antioxidants. High- or low-redness groups identify those birds over or below the median value of rump redness at the start of the experiment. Least square means ± SEs from models. LSD pairwise tests: **$P < 0.01$, *$P > 0.05$; $^{\dagger}P < 0.10$. Cohen's *d* effect sizes in Table S4.**

low-redness mitoTEMPO-treated birds might reveal their inefficiency (a delayed effect) to rapidly activate the hypothesized mitochondrial-based mechanism. This would also be coherent with the SPH framework. In this regard, we found that the interaction effect on feathers and color (Fig. 4) remained unaltered when the plasma 3HOE level was tested as a covariate (see Supporting Information). This means that the results may be independent of plasma carotenoid variability at the sampling date, being probably a consequence of blood ketocarotenoid levels that occurred days before sampling.

The results, as a whole, reveal a link between red ketocarotenoid-based coloration and individual quality mediated by the mitochondrial redox machinery. This is because mito-targeted antioxidants affected both ketocarotenoid levels and color. The link to mitochondrial function may give new support to the SPH (Hill 2011; Johnson and Hill 2013). It also suggests that red ketocarotenoid-based colorations could have evolved as a signaling system in different taxa due to its reliability in transmitting information. Thus, it has been suggested that dietary carotenoid transformation might reinforce the association

between coloration and individual quality, being probably a relevant mechanism in sustaining signal reliability (McGraw 2006; Weaver et al. 2018). Accordingly, it has recently reported that red ketocarotenoid-based ornaments in avian taxa are more strongly correlated to individual condition that those colorations produced by non-transformed pigments (Weaver et al. 2018). Now, studies simultaneously testing mitochondrial and ketolase activities in model animal species with ketocarotenoid-based ornamentation are needed to fully disentangle the mechanism involved in signaling and its ultimate evolutionary consequences.

AUTHOR CONTRIBUTIONS

C.A.A. and A.C. carried out the experiment, analyzed data, and drafted the manuscript. C.A.A. designed the study. D.A. and B.F.E. were responsible for crossbill captures and data measurements in the field. P.C., R.M., and A.C. were involved in HPLC laboratory analyses. All authors approve the publication and are accountable for this work.

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DATA ARCHIVING

All data will be available at DIGITAL.CSIC repository (https://digital.csic.es/). All data will be available at the DIGITAL.CSIC repository (http://doi.org/10.20350/digitalCSIC/12585).

LITERATURE CITED

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

Alonso-Alvarez, C., E. García-de Blas, and R. Mateo. 2018. Dietary canthaxanthin reduces xanthophyll uptake and red coloration in adult red-legged partridges. J. Exp. Biol. 221:jeb185074.

Alonso-Alvarez, C., S. Bertrand, G. Devevey, M. Gaillard, J. Prost, B. Faivre, and G. Sorci. 2004. An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. Am. Nat. 164:651–659.

Allen, R. M., and K. C. Vickers. 2014. Coenzyme Q10 Increases Cholesterol Efflux and Inhibits Atherosclerosis Through MicroRNAs. Arterioscler Thromb Vasc Biol. 34:1795–1797.

Biernaskie, J. M., A. Grafen, and J. C. Perry. 2014. The evolution of index signals to avoid the cost of dishonesty. Proceedings of the Royal Society B 281:20140876.

Blom, G. 1958. Statistical estimates and transformed beta-variables. Wiley, New York, NY.

Blount, J. D., M. P. Speed, G. D. Ruxton, and P. A. Stephens. 2009. Warning displays may function as honest signals of toxicity. Proceedings of the Royal Society B: Biological Sciences 276:871–877.

Braendle, C., A. Heyland, and T. Flatt. 2011. Integrating mechanistic and evolutionary analysis of life history variation. Pp. 3-10 *in* T. Flatt, A. Heyland, eds. Mechanisms of life history evolution, Oxford Univ. Press, Oxford, New York.

Britton, G., S. Liaaen-Jensen, and H. Pfander. 2004. Carotenoids handbook. Birkhäuser, Basel.

Cantarero, A., L. Pérez-Rodréguez, A. A. Romero-Haro, O. Chastel, and Alonso-Alvarez. 2019. Carotenoid-based coloration predicts both longevity and lifetime fecundity in male birds, but testosterone disrupts signal reliability. Plos One 14:e0221436.

Cantarero, A., and C. Alonso-Alvarez. 2017. Mitochondria-targeted molecules determine the redness of the zebra finch bill. Biol. Lett. 13:20170455.

Cohen, J. 1992. A power primer. Psychol. Bull. 112:155–159.

Darwin, C. 1871. The descent of man, and selection in relation to sex. John Murray, Albermarle Street, London.

del Val, E., A. Borras, J. Cabrera, and J. C. Senar. 2009a. Plumage colour of male Common Crossbills *Loxia curvirostra*: visual assessment validated by colorimetry. Revista Catalana d'Ornitologia 25:19–25.

del Val, E., J. J. Negro, J. Garrido-Fernández, M. Jarén, A. Borràs, J. Cabrera, and J. C. Senar. 2014. Seasonal variation of red carotenoid pigments in plasma of wild Crossbill males *Loxia curvirostra*. J. Ornithology 155:211-218.

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

Dikalova, A. E., A. T. Bikineyeva, K. Budzyn, R. R. Nazarewicz, L. McCann, W. Lewis, D. G. Harrison, and S. I. Dikalov. 2010. Therapeutic Targeting of Mitochondrial Superoxide in Hypertension. Circ. Res. 107:106-116.

Doughan, A. K., and S. I. Dikalov. 2007. Mitochondrial redox cycling of mitoquinone leads to superoxide production and cellular apoptosis. Antioxidants and Redox Signaling 9:1825–1836.

Emlen, D. J., I. A. Warren, A. Johns, I. Dworkin, and L. C. Lavine ç. 2012. A mechanism of extreme growth and reliable signaling in sexually selected ornaments and weapons. Science 337:860–864.

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

Fink, B. D., D. F. Guo, C. A. Kulkarni, K. Rahmouni, R. J. Kerns, and W. I. Sivitz. 2017. Metabolic effects of a mitochondrial-targeted coenzyme Q analog in high fat fed obese mice. Pharmacology Research & Perspectives 5:e00301.

Fitze, P. S., and H. Richner. 2002. Differential effects of a parasite on ornamental structures based on melanins and carotenoids. Behavioral Ecology 13:401–407.

Foley, J. D., and A. Van Dam. 1982. Fundamentals of interactive computer graphics. Addison-Wesley, Reading, MA.

Fox, D. L., A. A. Wolfson, and J. W. McBeth. 1969. Metabolism of β-carotene in the American flamingo, *Phoenicopterus ruber*. Comp. Biochem. Physiol. 29:1223–1229.

Galván, I., K. Wakamatsu, P. R. Camarero, R. Mateo, and C. Alonso-Alvarez. 2015. Low-quality birds do not display high-quality signals: The cysteine-pheomelanin mechanism of honesty. Evolution 69:26–38.

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

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

Ge, Z., J. D. Johnson, P. A. Cobine, K. J. McGraw, R. Garcia, and G. E. Hill. 2015. High concentrations of ketocarotenoids in hepatic mitochondria of *Haemorhous mexicanus*. Physiol. Biochem. Zool. 88:444–450.

Gómez, J., and G. Liñán-Cembrano. 2017. SpotEgg: an image-processing tool for automatised analysis of colouration and spottiness. J. Avian Biol. 48:502–512.

Gottwald, E. M., M. Duss, M. Bugarski, D. Haenni, C. D. Schuh, E. M. Landau, and A. M. Hall. 2018. The targeted anti-oxidant MitoQ causes mitochondrial swelling and depolarization in kidney tissue. Physiol. Rep. 6:e13667.

Grafen, A. 1990. Biological signals as handicaps. J. Theor. Biol. 144:517–546.

Grether, G. F., J. Hudon, and D. F. Millie. 1999. Carotenoid limitation of sexual coloration along an environmental gradient in guppies. Proc. R. Soc. B: Biol. Sci. 266:1317–1317.

Gutiérrez-Tenorio, J., G. Marín-Royo, E. Martínez-Martínez, R. Martín, M. Miana, N. López-Andrés, R. Jurado-López, I. Gallardo, M. Luaces, J. A. San Román, et al. 2017. The role of oxidative stress in the crosstalk between leptin and mineralocorticoid receptor in the cardiac fibrosis associated with obesity. Sci. Rep. 7:16802.

Gonzalez, Y., B. Aryal, L. Chehab, and V. A. Rao. 2014. Atg7- and Keap1-dependent autophagy protects breast cancer cell lines against mitoquinone-induced oxidative stress. Oncotarget 5:1526–1537.

Hadfield, J. D., and I. P. F. Owens. 2006. Strong environmental determination of a carotenoid-based plumage trait is not mediated by carotenoid availability. J. Evol. Biol 19:1104–1114.

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

Hasson, O. 1997. Towards a general theory of biological signaling. J. Theor. Biol. 185:139–156.

Hill, G. E. 1990. Female house finches prefer colourful males: sexual selection for a condition-dependent trait. Anim. Behav. 40:563–572.

———. 1994. Geographic-variation in male ornamentation and female mate preference in the house finch - a comparative test of models of sexual selection. Behavioral Ecology 5:64–73.

———. 2011. Condition-dependent traits as signals of the functionality of vital cellular processes. Ecology Letters 14:625–634.

Hill, G. E., W. R. Hood, Z. Ge, R. Grinter, C. Greening, J. D. Johnson, N. R. Park, H. A. Taylor, V. A. Andreasen, M. J. Powers, et al. 2019. Plumage redness signals mitochondrial function in the house finch. Proc. Royal Society B: Biological Sciences 286:20191354.

Hill, G. E., C. Y. Inouye, and R. Montgomerie. 2002. Dietary carotenoids predict plumage coloration in wild house finches. Proc. Royal Society of London B: Biological Sciences 269:1119–1124.

Hill, G. E., and L. S. Johnson. 2012. The vitamin A-redox hypothesis: a biochemical basis for honest signaling via carotenoid pigmentation. Am. Nat. 180:E127–150.

Hu, Q., J. Ren, G. Li, J. Wu, X. Wu, G. Wang, G. Gu, H. Ren, Z. Hong, and J. Li. 2018. The mitochondrially targeted antioxidant MitoQ protects the intestinal barrier by ameliorating mitochondrial DNA damage via the Nrf2/ARE signaling pathway. Cell Death. Dis. 9:403.

Hudon, J. 1994. Showiness, carotenoids, and captivity: a comment on Hill (1992). The Auk 111:218–221.

Inouye, C. Y., G. E. Hill, R. D. Stradi, and R. Montgomerie. 2001. Carotenoids pigment in male house finch plumage in relation to age, subspecies, and ornamental coloration. The Auk 118:900–915, 916.

Jenni, L., and R. Winkler. 1994. Moult and ageing of European passerines, Academic Press, London.

Jeong, E.-M., J. Chung, H. Liu, Y. Go, S. Gladstein, A. Farzaneh-Far, E. D. Lewandowski, and S. C. Dudley. 2016. Role of Mitochondrial Oxidative Stress in Glucose Tolerance, Insulin Resistance, and Cardiac Diastolic Dysfunction. J. Am. Heart Assoc 5:e003046.

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

Koch, R. E., and G. E. Hill. 2018. Do carotenoid-based ornaments entail resource trade-offs? An evaluation of theory and data. Functional Ecology 32:1908-1920.

Koch, R. E., M. Staley, A. N. Kavazis, D. Hasselquist, M. B. Toomey, and G. E. Hill. 2019. Testing the resource trade-off hypothesis for carotenoid-based signal honesty using genetic variants of the domestic canary. J. Exp. Biol. 222:jeb188102.

Kodric-Brown, A. 1985. Female preference and sexual selection for male coloration in the guppy (*Poecilia reticulata*). Behav. Ecol. Sociobiol. 17:199–205.

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

Lopes, R. J., J. D. Johnson, M. B. Toomey, M. S. Ferreira, P. M. Araujo, J. Melo-Ferreira, L. Andersson, G. E. Hill, J. C. Corbo, and M. Carneiro. 2016. Genetic basis for red coloration in birds. Curr. Biol. 26:1427–1434.

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

Maroz, A., R. F. Anderson, R. A. J. Smith, and M. P. Murphy. 2009. Reactivity of ubiquinone and ubiquinol with superoxide and the hydroperoxyl radical: implications for in vivo antioxidant activity. Free Radic. Biol. Med. 46:105–109.

Maynard Smith, J., and D. Harper. 2003. Animal signals (Oxford Series in Ecology and Evolution). Oxford University Press, Oxford, U.K. https://global.oup.com/academic/product/animal-signals-9780198526858?lang=en&cc=es.

McGraw, K. J. 2006. Mechanics of carotenoid-based coloration. in G. E. Hill and K. J. McGraw, eds. Bird coloration, Volume 1: Mechanisms and measurements. Harvard University Press, Harvard.

McGraw, K. J., G. E. Hill, and R. S. Parker. 2003. Carotenoid pigments in a mutant cardinal: implications for the genetic and enzymatic control mechanisms of carotenoid metabolism in birds. The Condor 105:587–592.

McGraw, K. J., and M. B. Toomey. 2010. Carotenoid accumulation in the tissues of zebra finches: predictors of integumentary pigmentation and implications for carotenoid allocation strategies. Physiol. Biochem. Zool. 83:97–109.

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

Mortensen, A., and L. H. Skibsted. 1997. Importance of Carotenoid Structure in Radical-Scavenging Reactions. J. Agric. Food Chem. 45:2970–2977.

Mougeot, F., J. Martinez-Padilla, L. Perez-Rodriguez, and G. R. Bortolotti. 2007. Carotenoid-based colouration and ultraviolet reflectance of the sexual ornaments of grouse. Behav. Ecol. Sociobiol. 61:741–751.

Mundy, N. I., J. Stapley, C. Bennison, R. Tucker, H. Twyman, K.-W. Kim, T. Burke, T. R. Birkhead, S. Andersson, and J. Slate. 2016. Red carotenoid coloration in the zebra finch is controlled by a cytochrome P450 gene cluster. Curr. Biol. 26:1435–1440.

Murphy, M. P., and R. A. Smith. 2007. Targeting antioxidants to mitochondria by conjugation to lipophilic cations. Annu. Rev. Pharmacol. Toxicol. 47:629-656.

Nazarewicz, R. R., A. Dikalova, A. Bikineyeva, S. Ivanov, I. A. Kirilyuk, I. A. Grigor'ev, and S. I. Dikalov. 2013. Does scavenging of mitochondrial superoxide attenuate cancer prosurvival signaling pathways? Antioxid. Redox Signaling 19:344–349.

Panda, A. K., and G. Cherian. 2014. Role of vitamin E in counteracting oxidative stress in poultry. The Journal of Poultry Science 51:109–117.

Penn, D. J., and S. Számadó. 2020. The Handicap Principle: how an erroneous hypothesis became a scientific principle. Biological Reviews. https://doi.org/10.1111/brv.12563.

Reby, D., and K. McComb. 2003. Anatomical constraints generate honesty: acoustic cues to age and weight in the roars of red deer stags. Anim. Behav. 65:519–530.

Reily, C., T. Mitchell, B. K. Chacko, G. A. Benavides, M. P. Murphy, and V. M. Darley-Usmar. 2013. Mitochondrially targeted compounds and their impact on cellular bioenergetics. Redox. Biol. 1:86–93.

Ridout, M. S., C. Demétrio, and J. Hinde. 1998. Models for count data with many zeros. Proceedings of the XIXth International Biometric Conference, 179–192. Cape Town.

Rodríguez-Estival, J., M. Martínez-Haro, M. P. Martín-Hernando, and R. Mateo. 2010. Sub-chronic effects of nitrate in drinking water on red-legged partridge (Alectoris rufa): Oxidative stress and T-cell mediated immune function. Environ. Res. 110:469–475.

Reszczynska, E., R. Welc, W. Grudzinski, K. Trebacz, and W. I. Gruszecki. 2015. Carotenoid binding to proteins: Modeling pigment transport to lipid membranes. Arch. Biochem. Biophys 584:125–133.

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

Smith, R. A. J., C. M. Porteous, A. M. Gane, and M. P. Murphy. 2003. Delivery of bioactive molecules to mitochondria in vivo. Proc. Natl. Acad. Sci. U. S. A 100:5407–5412.

Svensson, P. A., and B. Wong. 2011. Carotenoid-based signals in behavioural ecology: A review. Behaviour 148:131–189.

Toews, D. P. L., N. R. Hofmeister, and S. A. Taylor. 2017. The evolution and genetics of carotenoid processing in animals. Trends Genet. 33:171–182.

Trnka, J., F. H. Blaikie, R. A. J. Smith, and M. P. Murphy. 2008. A mitochondria-targeted nitroxide is reduced to its hydroxylamine by ubiquinol in mitochondria. Free Radic. Biol. Med. 44:1406–1419.

Trnka, J., M. Elkalaf, and M. Anděl. 2015. Lipophilic triphenylphosphonium cations inhibit mitochondrial electron transport chain and induce mitochondrial proton leak. PLoS One 10:e0121837.

Vendrov, A. E., K. C. Vendrov, A. Smith, J. Yuan, A. Sumida, J. Robidoux, M. S. Runge, and N. R. Madamanchi. 2015. NOX4 NADPH oxidase-dependent mitochondrial oxidative stress in aging-associated cardiovascular disease. Antioxid. Redox Signaling 23:1389–1409.

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

von Schantz, T., S. Bensch, M. Grahn, D. Hasselquist, and H. Wittzell. 1999. Good genes, oxidative stress and condition-dependent sexual signals. Proc. R. Soc. B-Biol. Sci. 266:1–12.

Wallace, A. R. 1877. The Colors of Animals and Plants. Am. Nat. 11:641–662.

Wang, A., A. V. Keita, V. Phan, C. M. McKay, I. Schoultz, J. Lee, M. P. Murphy, M. Fernando, N. Ronaghan, D. Balce, et al. 2014. Targeting mitochondria-derived reactive oxygen species to reduce epithelial barrier dysfunction and colitis. Am. J. Pathol. 184:2516–2527.

Wang, Y., and S. Hekimi. 2016. Understanding ubiquinone. Trends Cell Biol. 26:367–378. https://doi.org/10.1016/j.tcb.2015.12.007.

Weaver, J., R. K.och. R., and G. E. Hill. 2017. What maintains signal honesty in animal color displays? Philosophical Trans. Royal Society B 372:20160343.

Weaver, R. J., E. S. A. Santos, A. M. Tucker, A. E. Wilson, and G. E. Hill. 2018. Carotenoid metabolism strengthens the link between feather coloration and individual quality. Nat. Commun. 9:73.

Weber, H. 1961. Über die Ursache des Verlustes der roten Federfarbe bei gekäfigten Birkenzeisigen. Journal für Ornithologie 102:158–163.

Yasui, H., K. Yamamoto, M. Suzuki, Y. Sakai, T. Bo, M. Nagane, E. Nishimura, T. Yamamori, T. Yamasaki, and K.-I. Yamada. 2017. Lipophilic triphenylphosphonium derivatives enhance radiation-induced cell killing via inhibition of mitochondrial energy metabolism in tumor cells. Cancer Lett. 390:160–167.

Zielonka, J., J. Joseph, A. Sikora, M. Hardy, O. Ouari, J. Vasquez-Vivar, G. Cheng, M. Lopez, and B. Kalyanaraman. 2017. Mitochondria-Targeted Triphenylphosphonium-Based Compounds: Syntheses, Mechanisms of Action, and Therapeutic and Diagnostic Applications. Chem. Rev. 117:10043–10120.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Molecular structure of the two mito-targeted antioxidants and their situation into the inner mitochondrial membrane and electron transport chain.
Table S1. Sample size distribution depending on the age categories (8-12 months old vs. older birds; i.e., following Jenni and Winkler 1994, see also main text).
Table S2. Descriptive statistics of carotenoid and vitamin concentrations in plasma and feathers calculated from the entire captive population.
Table S3. Chromatographic information of the five unidentified xanthophylls (UX) found in the blood plasma of captive red crossbills.
Fig. S2. Diagram illustrating the chemical structures and relevant conversion pathways of two dietary carotenoids (β-cryptoxanthin, and b-carotene) into the metabolically transformed red ketocarotenoids detected in red crossbills (3-hydroxy-echinenone, echinenone and canthaxanthin).
Fig. S3. Image of one male red crossbill where the difference in color between original and regrowth rump feathers is well appreciated.

SUPPLEMENTARY INFORMATION

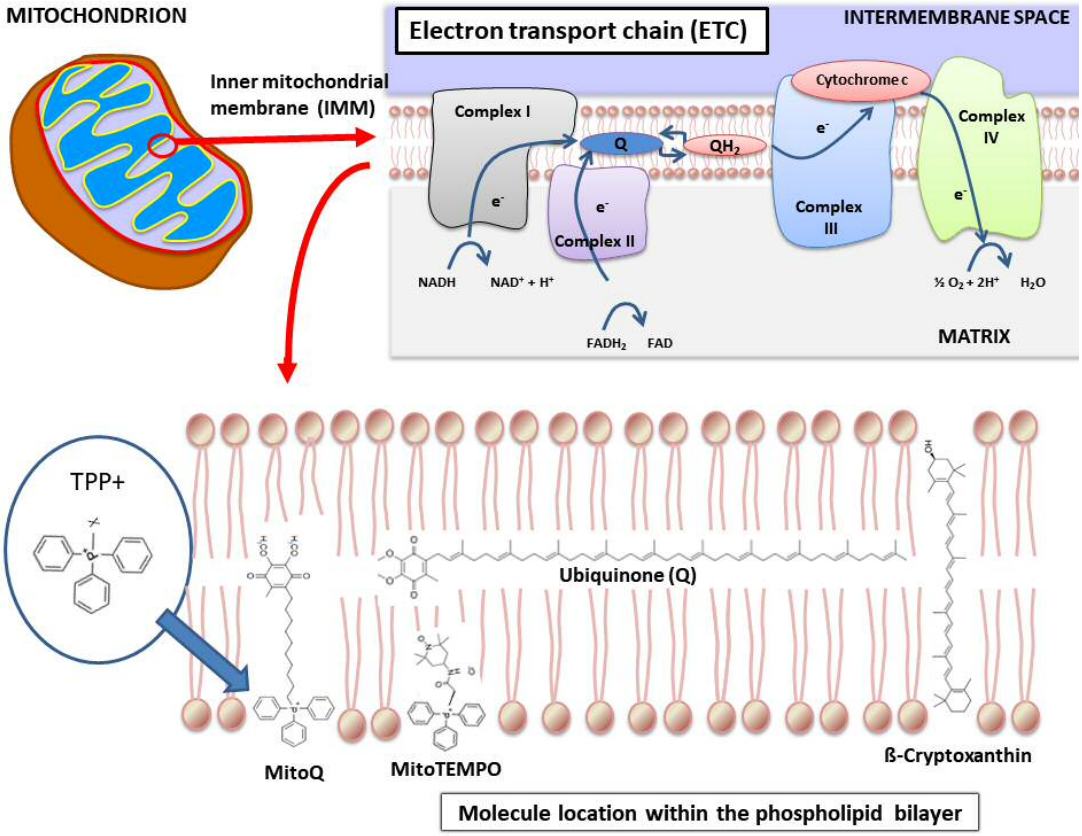

Fig. S1. Molecular structure of the two mito-targeted antioxidants and their situation into the inner mitochondrial membrane and electron transport chain.

Carotenoid supplement

Peanut oil was used because it has previously been described in avian studies manipulating dietary carotenoids (e.g. Saino et al. 2000) and because it allows dilution without adding substantial carotenoid amounts (0.1 mg/kg in crude oil; Carrín and Carelli 2010). the result being mixed by a vortex and also sonication (30 s) in cold water. The synthetic β-carotene solution was diluted in peanut oil (SIGMA-ALDRICH ref. P2144) at 1:100 volumes to reduce its very high concentration. The dry mandarin extract was then added to that solution (19.3 g per 100 mL; i.e. the highest concentration that we were able to dilute). The

mixture was maintained refrigerated and away from light and regularly made to avoid oxidation (Alonso-Alvarez et al. 2004).

Testing potential initial biases in body mass, size or condition

Besides being balanced by treatment and color category (main text), the sample size was also equilibrated based on body mass or size variability at the beginning of the experiment. Accordingly, the treatment, color category and interaction did not report a significant influence on tarsus length, body mass, or size-corrected body mass (tarsus length as a covariate) at the date of first injection (all tests: *P*-values > 0.23).

Age groups among treatments and color categories

As mentioned in the precedent section and main text, we aimed to equally distribute the sample size among treatments and color categories, but also balancing body mass, size and condition to avoid subtle initial biases. This, however, led to some bias in the distribution of the two age categories (8-12-month-old vs. older birds) among mitoQ-treated birds (Table S1 below). The age category would be balanced between the color categories in both controls (Fisher's two-tailed exact test $P = 0.301$) and mitoTEMPO-birds ($P = 0.580$). However, a trend to significance was found among mitoQ-birds ($P = 0.091$). Here, low-redness birds only included an individual older than one year (Table S1). Moreover, when considering the absence of some few data in some variables (i.e., 37, 36 and 35 for color, feather and plasma analyses, respectively; cited in the main text), the detected unbalance only tended to significance in plasma analyses (i.e., $P > 0.061$; other P's > 0.10).

Table S1. Sample size distribution depending on the age categories (8-12 months old vs. older birds; i.e., following Jenni and Winkler 1994, see also main text).

		High-redness	Low-redness	Total
Control	Younger	4	4	**8**
	Older	4	2	**6**
	Total	**8**	**6**	**14**
MitoTEMPO	Younger	3	5	**8**
	Older	3	3	**6**
	Total	**6**	**8**	**14**
MitoQ	Younger	2	7	**9**
	Older	4	1	**5**
	Total	**6**	**8**	**14**
	Total	**20**	**22**	

Rate of follicle development

On June 7, that is, five days before the second (final) blood sampling, a digital picture of the rump of each bird was taken. The birds were then classified with a feather development score (0-6), from no follicle sign in the epidermis (0 value) to follicles well-distributed over the full rump surface and showing some barbule emergence (6 value). The classification was made by CAA blind to experimental treatment classifications. Only one bird did not show growing follicles, which was the same individual that finally did not produce rump feathers (main text). Among blood sampled birds (see Discussion), the 86% birds showed well-formed colored follicles in most of the rump surface (3-6). The experimental treatment factor, color category, and its interaction did not report any significant effect (all $P > 0.35$; PROC GENMOD in SAS 9.4, multinomial distribution with cumlogit link). Taking into account that another five days passed to blood extraction, we may assume that most birds ended the phase in which blood carotenoids could be allocated to follicles when they were blood sampled (see Discussion).

Color analysis

A digital picture of each bird was taken (see also details in Methods). A Kaiser Repro Base (Kaiser Fototechnik, Buchen) was used, including a gridded board and a column to place the camera at the same height (distance from the board to the lens: 38 cm). The base was covered with opaque grey cellular polycarbonate sheets placed in a vertical position to cover the four sides of the board. It allowed us to enter the camera objective and ring flash (Canon Macro Ring Lite MR-14EX) from the top. The sheets were perforated to allow entering the hands of a person that would hold the body of the bird resting on the board surface. All the open surfaces were covered with PVC blackout fabric to reduce the light on the board surface. The focus and diaphragm of the camera and the ring flash were all manually fixed to avoid the interference of automatic functions.

Carotenoid analysis by HPLC

Carotenoids and vitamins A and E in the rump feathers and plasma were analyzed by HPLC (Agilent Technologies 1200 series) with DAD and FLD detectors (main text). Compound separation required a column Zorbax Eclipse XBD-C18 (4.6x150 mm, 5 µm) with a precolumn Zorbax Eclipse XBD-C18 (4.6x10 mm, 5 µm). The injection volume was 20 µL. The chromatographic conditions consisted of gradient elution of two phases (A: formic acid 0.1 % in water; B: formic acid 0.1 % in methanol). The initial conditions were 50 % A and 50 % B, changing to 0 % A and 100 % B in 20 minutes, keep this condition 30 minutes and return to initial conditions in 5 minutes, all time the flow rate was 1 mL/min.

Carotenoid extraction from feathers

To avoid contamination of carotenoids from uropygium secretion, the feathers were washed with ethanol, hexane, and then air-dried. Then, the feathers were extracted in a mixer mill with grinding steel balls (MM400, Retsch, Germany) for 3 min with 5 mL of methanol and 50 µl of internal standard (retinyl acetate at 0.5 mM). Subsequently, the extract was filtered through a nylon syringe filter 0.2µm, dried under N2 stream and finally re-suspended with 200 µl of ethanol and transferred to a chromatographic vial for HPLC analysis.

Table S2. Descriptive statistics of carotenoid and vitamin concentrations in plasma and feathers calculated from the entire captive population.

First blood sample (nmol/mL)	Min	Max	Mean	SD
Retinol	2.70	8.54	5.28	1.34
Total Tocopherol	112.10	323.03	197.30	51.50
Lutein	6.35	81.86	26.03	12.43
β-cryptoxanthin	0	.78	.32	.15
Unidentified Xanthophylls (total)	2.30	17.10	6.01	2.80
Canthaxanthin	0	2.056	.48	.74
3-hydroxyechinenone (3HOE)	0	.86	.18	.21
Echinenone	0	.15	.01	.03
Total yellow carotenoids	8.94	99.22	32.36	15.22
Total red ketocarotenoids	0	2.67	.67	.85

Second blood sample (nmol/mL)	Min	Max	Mean	SD
Retinol	3.14	9.98	5.72	1.45
Total tocopherol	0	383.56	189.25	64.71
Lutein	7.91	50.12	20.56	9.89
β-cryptoxanthin	0	1.06	.47	.26
Unidentified Xanthophylls (total)	1.74	12.64	5.18	2.37
Astaxanthin	0	.51	.05	.10
Canthaxanthin	0	1.89	.52	.66
3-hydroxyechinenone (3HOE)	0	3.42	.50	.72
Echinenone	0	.27	.04	.06
Total yellow carotenoids	10.52	63.35	26.21	12.27
Total red ketocarotenoids	0	5.73	1.11	1.22

Rump feathers (nmol/g)	Min	Max	Mean	SD
Astaxanthin	0	5	.16	.867
Lutein	.51	180.29	75.45	38.46
Canthaxanthin	0	17	4.89	3.49
3-hydroxyechinenone (3HOE)	0	74	16.84	20.23
UD-β-cryptoxanthin	0	17	4.60	3.64
β-cryptoxanthin	0	11	2.57	2.16
Echinenone	0	4	.78	.882
Total yellow carotenoids	1.60	190.99	82.62	39.84
Total red ketocarotenoids	0	78	22.51	20.32

Unidentified carotenoids in plasma and feathers

Five unidentified carotenoids (here identified as C1 to C5) were found in blood plasma. Their wavelength of maximum absorbance (λmax) and retention time (RT) suggested that they were close to yellow xanthophylls such as lutein or β-cryptoxanthin (Table S3). Also, an unidentified form (probably an isomer) of β-cryptoxanthin was found in feathers (RT: 36.12 min, λmax: 445 nm). Note that all the readings in the analyses were done at 476 nm.

Table S3. Chromatographic information of the five unidentified xanthophylls (UX) found in the blood plasma of captive red crossbills.

	C1	C2	C3	C4	C5
RT (min)	23.26	23.90	25.05	25.53	31.23
λmax (nm)	445	445	445	450	445

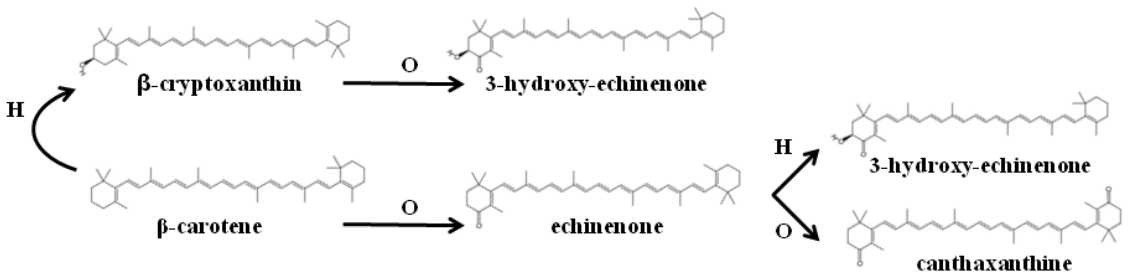

Fig. S2. Diagram illustrating the chemical structures and relevant conversion pathways of two dietary carotenoids (β-cryptoxanthin, and b-carotene) into the metabolically transformed red ketocarotenoids detected in red crossbills (3-hydroxy-echinenone, echinenone and canthaxanthin). The letters above the arrows indicate the nature of the reaction undergone: H = hydroxylation, and O = oxidation (following McGraw 2006, p. 215).

115 **Color categories**

116 In addition to digital picture measurements of rump redness, the full plumage was visually
117 assigned to four color categories (yellow, patchy-orange, uniform orange or red) following
118 del Val et al. (2014). This was made by DA on the day of capture and again one week after.
119 These two classifications were highly correlated (Spearman's r = 0.96, N = 47). The
120 number of birds in the red category was also half of the sample (23 individuals) and
121 matched at 90% with the red rump classification. We decided to use the rump categories as
122 it is the trait manipulated (plucked) here and the most intensely colored area in the species.

123

124 **Occurrence/absence of pigments in carotenoid-supplied birds vs. controls.**

125 Non-parametric χ^2 tests were used to test the absence/occurrence of pigments in the
126 comparison of dietary carotenoid supplement groups. Since the control group was at the
127 borderline of the criterion of minimum sample size for χ^2 tests in contingency tables (n =
128 5), P-values from Fisher's exact tests were also studied. Both tests reported similar results.

129 In plasma, canthaxanthin and echinenone were absent in controls at the first
130 sampling (note that the supplement was provided ten days before), but all χ^2 or Fisher's
131 exact tests showed $P > 0.14$. In the second sample, only the absence of circulating
132 echinenone in controls tended to significance in χ^2 tests (χ^2 = 3.49, df= 1, P = 0.062), but
133 not in the Fisher's exact test (P = 0.138).

134 In feathers, the difference between the two groups of birds was highly significant in
135 the case of β-cryptoxanthin (Pearsons' χ^2 = 20.25, P <0.001; Fisher's test: $P < 0.001$) and
136 echinenone (Pearsons' χ^2 = 8.04, P = 0.005, Fisher's test: P = 0.008). These two
137 carotenoids were absent in birds supplied with peanut oil only (controls). The absence rate
138 of UD-beta-cryptoxanthin in feathers was also higher among control birds (60%) compared
139 to carotenoid-supplied animals (13.9%; Pearsons' χ^2 = 5.94, P= 0.015; Fisher's test P =
140 0.043). The absence of canthaxanthin (40%) in the feathers from control birds was
141 significantly higher than that reported in birds receiving the supplement (6%; Pearsons' χ^2
142 = 5.92, df= 1, P = 0.016). However, the Fisher exact test only reported a trend to

significance ($P = 0.066$). No other feather pigment differed in absence/occurrence (all χ^2 or Fisher tests P-values > 0.90).

The appearance of new feathers

Regrowth feathers in the rump never attained the intense red color that birds can produce in the wild. The mean total amount of red ketocarotenoids in feathers was thus the 22% of total carotenoids in the whole of the birds' sample (Table S2). Note that color analyses were performed only on the surface of the rump that showed yellow-to-red coloration (avoiding grey parts).

Fig. S3. Image of one male red crossbill where the difference in color between original and regrowth rump feathers is well appreciated.

Covariation between blood 3HOE levels and coloration

We added plasma 3HOE values at the last sampling or, alternatively, the difference between final and initial 3HOE plasma levels, as covariates in the models testing feather redness or ketocarotenoid concentration variability. These covariates were always significantly and positively correlated to redness and plumage ketocarotenoid levels (always $P < 0.002$; slopes ranging from +0.33 ± 0.04 to +0.89 ± 0.11). However, the interaction in the redness model (Table 2 in the main text) and the comparison between high-redness mitoTEMPO-birds and high-redness controls in the feather ketocarotenoid model remained equally significant (all $P < 0.02$).

Cohen's *d* effect sizes

Table S4. Mean difference, SE and effect sizes for the significant pairwise comparisons of least squared means obtained from models testing the levels of carotenoids, vitamins and plumage redness (see Tables and Figs in the main text).

		Mean difference	SE difference	Cohen's *d*
Plasma β-cryptoxanthin				
	mitoQ vs CONTROL	0.244	0.060	1.7
	mitoQ vs mitoTEMPO	0.332	0.061	2.72
Plasma lutein				
	mitoQ vs CONTROL	5.894	2.729	0.9
	mitoQ vs mitoTEMPO	7.725	2.674	1.21
Plasma UX				
	mitoQ vs CONTROL	1.418	0.662	0.89
	mitoQ vs mitoTEMPO	1.506	0.658	0.95
Total tocopherol				
	mitoQ vs CONTROL	73.771	16.285	1.89
	mitoQ vs mitoTEMPO	47.578	16.334	1.22
Plasma canthaxanthin				
	mitoTEMPO vs CONTROL	0.388	0.142	1.12
	mitoTEMPO vs mitoQ	0.448	0.173	1.08
Plasma 3HOE				
	mitoQ-lowRed vs mitoTEMPO-lowRed	1.817	0.541	1.87
	mitoQ-highRed vs mitoTEMPO-highRed	1.516	0.742	1.29
	mitoQ-highRed vs mitoTEMPO-lowRed	2.745	0.777	2.07
	mitoQ-highRed vs CONTROL-highRed	1.934	0.721	1.57
	mitoQ-lowRed vs CONTROL-highRed	1.006	0.446	1.25
	CONTROL-lowRed vs mitoTEMPO-lowRed	1.391	0.471	1.73
	mitoTEMPO-lowRed vs mitoTEMPO-highRed	1.229	0.556	1.29
Feather β-cryptoxanthin				
	mitoQ vs CONTROL	0.788	0.338	0.97
	mitoQ vs mitoTEMPO	1.005	0.338	1.19
Feather echinenone				
	mitoQ vs CONTROL	0.481	0.194	1.04
	mitoQ vs mitoTEMPO	0.609	0.173	1.44
Feather red ketocarotenoids				
	mitoTEMPO-highRed vs CONTROL-highRed	0.530	0.233	1.31
	mitoTEMPO-highRed vs mitoQ-lowRed	0.464	0.236	1.09
Rump redness				
	mitoTEMPO-highRed vs mitoQ-lowRed	1.722	0.582	1.65
	mitoTEMPO-highRed vs CONTROL-highRed	1.848	0.586	1.82
	mitoTEMPO-highRed vs mitoTEMPO-lowRed	1.458	0.592	1.37

Literature cited

Alonso-Alvarez, C., S. Bertrand, G. Devevey, M. Gaillard, J. Prost, B. Faivre, and G. Sorci. 2004. An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. The American Naturalist 164:651-659.

Carrín, M. E. and A. A. Carelli. 2010. Peanut oil: Compositional data. European Journal of Lipid Science and Technology 112:697-707.

del Val, E., J. J. Negro, J. Garrido-Fernández, M. Jarén, A. Borràs, J. Cabrera, and J. C. Senar. 2014. Seasonal variation of red carotenoid pigments in plasma of wild Crossbill males *Loxia curvirostra*. J. Ornithol. 155:211-218.

McGraw, K. J. 2006. Mechanics of carotenoid-based coloration, Cambridge: Harvard University Press.

Saino, N., P. Ninni, S. Calza, R. Martinelli, F. D. Bernard, and A. P. Møller. 2000. Better red than dead: carotenoid-based mouth coloration reveals infection in barn swallow nestlings. Proceedings of the Royal Society of London B 267:57-61.