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Testing the carotenoid-based sexual signalling mechanism by altering *CYP2J19* gene expression and colour in a bird species

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Ornaments can evolve to reveal individual quality when their production/maintenance costs make them reliable as ‘signals’ or if their expression level is intrinsically linked to condition by some unfalsifiable mechanism (indices). The latter has been mostly associated with traits constrained by body size. In red ketocarotenoid-based colorations, that link could, instead, be established with cell respiration at the inner mitochondrial membrane (IMM). The production mechanism could be independent of resource (yellow carotenoids) availability, thus discarding costs linked to allocation trade-offs. A gene coding for a ketolase enzyme (*CYP2J19*) responsible for converting dietary yellow carotenoids to red ketocarotenoids has recently been described. We treated male zebra finches with an antioxidant designed to penetrate the IMM (mitoTEMPO) and a thyroid hormone (triiodothyronine) with known hypermetabolic effects. Among hormone controls, MitoTEMPO downregulated *CYP2J19* in the bill (a red ketocarotenoid-based ornament), supporting the mitochondrial involvement in ketolase function. Both treatments interacted when increasing hormone dosage, indicating that mitochondria and thyroid metabolisms could simultaneously regulate coloration. Moreover, *CYP2J19* expression was positively correlated to redness but also to yellow carotenoid levels in the blood. However, treatment effects were not annulated when controlling for blood carotenoid variability, which suggests that costs linked to resource availability could be minor.

1. Introduction

The animal signalling theory proposes that traits involved in animal communication, such as many ornaments or songs, may evolve due to production/maintenance costs that prevent cheating by low-quality individuals [1] but also because the level of expression of the trait directly reveals individual quality (i.e. they cannot be faked; [2]). The first type of trait is often defined as a ‘signal’ of quality or also ‘costly signals’, whereas the second type of trait is usually known as an ‘index’ of that quality or also ‘index signals’ (e.g. [3,4]). The expression level of sexual costly signals should in some way correlate positively with reproductive success, but negatively to survival, due to some direct or indirect costs of trait production/maintenance. Instead, ‘index signals’ should positively correlate to both reproductive success and survival (i.e. no trade-off should exist; [5]), and costs are not required to maintain honesty (but see [6]). Most examples of index signals have been associated with traits

(e.g. deer antlers or certain calling traits) that depend or are positively correlated to body size (i.e. [2,7]).

However, a new quite different type of index signal has been proposed in the form of conspicuous colorations produced by red carotenoid pigments [8,9]. Red carotenoid-based ornaments are present in many vertebrates and have attracted much attention from evolutionary ecologists as its proximate production mechanisms are intriguingly complex and could challenge pre-existing animal signalling hypotheses (e.g. [10]). Such complexity is, however, becoming disentangled at the molecular level (e.g. [11–13]). The new findings in this regard are stimulating the formulation of new ideas to explain why these mechanisms have promoted the selection of red ornaments as reliable information transmitters.

Carotenoids are molecules whose chromophore generates yellow to red colorations [14] and are often considered important in maintaining animal homeostasis as antioxidants or immune-boosters [15]. However, most animal organisms cannot synthesize them from any non-carotenoid substrate and must be directly acquired with the diet [10]. Thus, when carotenoids are scarce in food, a trade-off between carotenoid allocation to coloration versus homeostasis could have promoted the evolution of carotenoid-based ornaments as costly signals (i.e. the resource allocation trade-off hypothesis of carotenoid-based signalling; e.g. [16]).

Nonetheless, some yellow-to-orange dietary carotenoids (e.g. lutein, zeaxanthin) can be transformed into redder (keto)carotenoids (e.g. astaxanthin, canthaxanthin) by enzymatic reactions in the animals' body [10,17,18]. This conversion has been well studied in the avian taxa. Many aquatic bird species can easily obtain red ketocarotenoids from their food as these pigments are abundant in aquatic invertebrate prey [10]. This allows them to allocate ketocarotenoids to ornaments without transformation. However, among terrestrial birds, ketocarotenoids are often scarce in food (particularly in vegetal food), and molecular mechanisms allowing the transformation of yellow to red pigments have evolved [10]. Currently, the only candidate gene implicated in this transformation and well supported by molecular studies is *CYP2J19* [12,13,19,20]. *CYP2J19* is a member of the cytochrome P450 family of enzymes—a family of genes often involved in the metabolism of toxicants but also hormone synthesis [21].

Before the discovery of *CYP2J19*, some researchers argued that the candidate enzyme or enzymes involved in the transformation of yellow to red carotenoids (ketolases) should be linked to mitochondrial activity [8,11]. They argued that some ketocarotenoids are molecularly similar to a crucial antioxidant involved in the cell respiratory chain (i.e. ubiquinone). This would imply that the ketolase could be part of the ubiquinone enzymatic biosynthesis pathway [11]. The transformation would, hence, be made in the inner mitochondrial membrane (IMM) probably sharing a biochemical pathway with cell respiration. This was subsequently defined as the 'mitochondrial function hypothesis' [3,22]. The theoretical link to the mitochondria metabolism led these authors [8,23] to propose that red ketocarotenoid-based ornaments evolved as 'indices' of individual quality (i.e. [2]) as they would be tightly linked to basic metabolic pathways such as cell respiration.

The description of *CYP2J19* could support this hypothesis if the enzyme is indeed placed at the IMM affecting cell respiration. The recent finding of relatively high levels of

ketocarotenoids at the IMM compared to other cell fractions is consistent with this scenario [24]. Moreover, the treatment of male zebra finches (*Taeniopygia guttata*) with synthetic ubiquinone (mitoQ) designed to penetrate the IMM [25] increased bill redness [26], which is a ketocarotenoid-based sexually selected ornament [25].

In the present study, we exposed male zebra finches to another synthetic mito-targeted antioxidant (i.e. mitoTEMPO), testing, for the first time, potential changes in bill *CYP2J19* expression levels. MitoTEMPO is a superoxide dismutase mimetic reducing mitochondrial reactive oxygen species (ROS) production without the collateral effects associated with mitoQ (see Methods and references therein). This experiment allowed us to test if mitochondria and *CYP2J19* function are indeed linked and if that connection affects trait expression (redness). Moreover, we aimed to go further by artificially increasing the level of the most active thyroid hormone (triiodothyronine; T3), testing its impact on both *CYP2J19* expression and redness. We should note that virtually nothing is known about the potential involvement of thyroid hormones in animal carotenoid-based ornaments, even when this hormone seems to be a key piece in the evolution of phenotypic plasticity among vertebrates (e.g. [27,28]). The circulating levels of thyroid hormones are deeply affected by environmental factors such as food availability or temperature (reviewed in [29]). These hormones control oxygen consumption and induce hypermetabolic effects mediated by changes in mitochondria metabolism [30–32]. High blood levels of thyroid hormones are commonly associated with higher oxidative stress, and higher ROS production, particularly mitochondrial superoxide production (reviewed e.g. in [33–36]). High oxidative stress may, in turn, exert an inhibitory effect on the expression and activity of CYP enzymes [37].

Accordingly, and also taking into account precedent results on mito-targeted antioxidant compounds (i.e. [26,38]), we predicted that mitoTEMPO should increase the *CYP2J19* expression level and bill redness. By contrast, high T3 levels should decrease them, both treatments interacting perhaps to cancel out their respective effects. Moreover, if ornaments evolved as index signals, theory predicts that trait expression should not depend on the resource levels (carotenoid substrates) required for enzymatic conversion (i.e. yellow carotenoids) because this could generate production costs derived from resource allocation trade-offs (e.g. [16,17]). Zebra finches only circulate yellowish-orange carotenoids in the blood ([39] also electronic supplementary material). Hence, we predict that treatments should not affect circulating carotenoid levels, and no correlation between them and bill redness or *CYP2J19* expression should exist. Alternatively, if treatments increase or decrease ROS generation, plasma carotenoid levels could change due to, respectively, increased or reduced carotenoid consumption for homeostasis maintenance, which should accordingly affect colour and gene expression.

2. Material and methods

(a) Experimental protocol

Eighty-six male zebra finches were housed in indoor aviary cages (more details in electronic supplementary material). Two birds were housed per cage (0.6 m × 0.4 m × 0.4 m). The pair was divided by a grille hindering physical contact. After an 8-day

acclimation period, body mass, respiratory frequency (breath rate) and bill colour were measured, and a blood sample was also taken. Five days after blood sampling, all the birds were randomly assigned to the treatments and received a subcutaneous silicone implant (OD 1.96 mm, ID 1.477 mm; Silastic® empty or filled with T3 (3,3',5-triiodo-L-thyronine; SIGMA ref. T2822; see also electronic supplementary material). The T3-filled implants were made of different lengths (6 mm, $n = 22$, 8 mm, $n = 21$, and 10 mm, $n = 21$; approximately 12, 18 and 24 mg of T3, respectively) to produce different dosages. Control birds ($n = 22$) received a 10 mm empty implant. The largest length was chosen considering recent studies in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*; [40,41]). Lower dosages were included considering that sparrows are heavier than finches (26 versus 15 g approx., respectively). The implants were put in two consecutive days due to time constraints. Thirty-four birds rejected the implant 2–7 days before the end of the experiment (mean $\pm$ s.d.: 3 ± 1.2 days). The treatment distribution among birds losing or maintaining the implant never differs (i.e. by testing both treatments separately or a single eight-level factor all χ^2 tests: $p > 0.59$). The number of days with implant also did not differ among treatments (Kruskal–Wallis or Mann–Whitney U tests: all p -values > 0.30). This covariate never produced a significant effect in models (all p -values > 0.10) and did not alter the results, being removed.

Each implant group (Control [C], 6 mm [T3-1], 8 mm [T3-2], 10 mm [T3-3]) was also divided by half (10–11 birds per group) to randomly assign the antioxidant treatment (Serum [S] or MitoTEMPO [MT]). MitoTEMPO (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride) is structurally comparable to mitoQ [42]. In both molecules, the antioxidant fraction is joined to a triphenylphosphonium cation (TPP⁺) designed to penetrate the IMM [25]. In mitoQ, TPP⁺ is connected to ubiquinone by a 10-carbon alkyl chain (i.e. decyl-TPP⁺). Unfortunately, the length of this chain seems to increase membrane permeability, inhibiting the electron transport chain (ETC) and rising superoxide radical generation [43–45]. Consistent with this, zebra finches only treated with decyl-TPP⁺ (i.e. without ubiquinone) developed paler bills than controls [26]. MitoTEMPO does not include that linker group, and the antioxidant role is played by piperidine nitroxide instead of ubiquinone [46]. Piperidine nitroxide recycles ubiquinol (the reduced ubiquinone form) to ubiquinone [46]. This action lowers mitochondrial superoxide radical concentration [42]. Moreover, we have recently found that mitoTEMPO can increase ketocarotenoid-based feather redness in males from another bird species (the red crossbill; *Loxia curvirostra*; [38]).

MitoTEMPO was administered to zebra finches by subcutaneous injections in the back. MitoTEMPO-treated birds received mitoTEMPO at 1.6 mg ml^{-1} in $50 \text{ } \mu\text{l}$ saline. MitoTEMPO controls received the same saline volume only. MitoTEMPO-treated birds received $80 \text{ } \mu\text{g}$ of mitoTEMPO every other day to a total of seven doses ($2.67 \text{ mg Kg}^{-1} \text{ day}^{-1}$). First injections were performed 2 days after the surgery to allow birds' recovery and ended 14 days after. The mitoTEMPO dosage was chosen from results described in mice [47], from a precedent pilot study in zebra finches, and also from the cited experiment in red crossbills ([38] and also electronic supplementary material). One bird (T3-2 and mitoTEMPO-treated) died of an unknown cause during the experiment.

Birds were weighed 6 days after the first injection (intermediate body mass). Blood samples, bill colour and body mass measures were again taken the day after the last injection (final values). The treatments were randomly distributed across the aviary (cage rows and columns). Nonetheless, the two birds in each cage belonged to the same antioxidant treatment. The cage identity was included as a random term in all statistical models to control for pseudo-replication (below).

Finally, the occurrence of a body feather moult was assessed as this is a recognizable effect of thyroid hormones in birds (e.g. [15,41]). It was visually established at the end of the experiment by the same observer (AC). Birds were classified as engaged or not in moult (49 and 36 birds, respectively, i.e. 57.6% versus 42.4%).

(b) Thyroid hormone analyses

T3 levels were assessed from plasma obtained in the last blood sampling event. Hormone levels were measured using species-independent ELISA kits (Arbor Assays, Ann Arbor, MI; ref. K056-H1). The analyses were made twice per sample in three sessions (intra- and inter-assay CVs = 10.2 and 14.8%, respectively; also electronic supplementary material).

(c) Breath rate

The frequency of breath of each bird was measured to validate the T3 impact on general metabolism (electronic supplementary material). This was made just before each blood sampling event by the same observer (AC). The number of breast movements in 90 s was counted by handing each bird face up in the left hand, with the head between the middle and forefinger [48].

(d) Plasma carotenoids

Total plasma carotenoid levels were determined at the start and end of the experiment through spectrophotometry. Samples were diluted in ethanol, centrifuged and supernatant absorbance measured at 450 nm. The concentrations were calculated from a lutein standard curve (electronic supplementary material).

(e) Gene expression

At the end of the experiment, a small layer of the upper surface of the upper mandible (1 mm^3 approx.) was taken with a small scalpel. The wound was disinfected and covered with blastoestimulina cream® (Almirall labs, Spain; composed of *Centella asiatica* extract plus neomycin). All birds fully recovered in less than five weeks. The biopsied tissue was immediately introduced in RNAlater at 1:20 volume approximately and stored frozen (-20°C) until the analyses. Total RNA was extracted using the RNeasy Mini Kit (Qiagen). Residual genomic DNA carry-over was removed using the DNase treatment from the same kit. Complementary DNA (cDNA) was prepared from total RNA (approx. $1 \text{ } \mu\text{g}$) using the GRS cDNA Synthesis Kit (GRiSP). Quantitative real-time PCR reactions were performed on the *CYP2J19* gene (target) on cDNA. Reactions were performed by using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories) in a CFX96 Touch Real-Time PCR Detection System. β -Actin was used as a housekeeping (control) gene for normalizing expression levels. Primers for both *CYP2J19* and β -actin were taken from a previous study (i.e. [12]). Mean cycle threshold (Ct) values of both genes were obtained from triplicated measures (both Lessells & Boag 1987' r values = 0.99). Expression values of target genes are traditionally corrected to the expression of the control gene using a ΔCt approach, but this assumes that the expression of the control gene is kept constant across conditions. Since our experiment is likely to influence overall homeostasis, and therefore impact control gene expression, we obtained normalized Ct values using the method of Cui *et al.* [49]: $\text{normalized value} = \text{target_Ct value} - (b \times \text{control_Ct value})$ where b is the regression coefficient of the linear regression of mean *CYP2J19* Ct values on mean β -actin Ct values. This normalization removes biases produced when the housekeeping Ct values correlate to $\Delta\text{-Ct}$ (i.e. *CYP2J19* Ct minus β -actin Ct; i.e. [49]).

(f) Colour measurements

Bill redness was determined using digital photography (Nikon® D300; full description in electronic supplementary material). Briefly, each bird was placed laterally, and a picture of one side of the head was taken. Digital photographs were standardized and analysed using the recently developed 'SpotEgg' software [50]. This is an image-processing tool for automatized analysis of avian coloration that solves the need for linearizing the camera's response to subtle changes in light intensity [51]. Mean red, green and blue (RGB) values measured from the lateral area of the bill (upper and lower mandibles) were used to calculate hue values following Foley & van Dam [52]. Repeatability calculated on a set of digital photographs measured twice ($n = 30$) was $r = 0.99$, $p < 0.001$. Since a low hue measure means a redder colour, the hue value was reversed ($\times -1$) to obtain a 'redness' variable (also electronic supplementary material).

(g) Statistical analyses

Dependent variables were tested by generalized linear mixed models in SAS (v. 9.4), with cage identity as a random term (electronic supplementary material). The treatments and their interaction were tested. The location of the bird in the cage (right versus left side) was also tested but removed when reported a $p > 0.10$ value (electronic supplementary material). Microplate identity was added as another random factor in models testing plasma T3 and carotenoid levels (all p -values > 0.22). Random factors were always maintained in the model (electronic supplementary material).

In models testing body mass differences among sampling events, the tarsus length was added to assess size-independent variability. Bill brightness was included in the bill redness model to avoid the influence of the total amount (intensity) of light reflected by the surface of the bill [10]. This would allow discarding the influence of non-pigmented tissue structures on redness [10]. The area selected for colour measurement was also tested as a covariate in the redness model. This model reported a similar result when that covariate was removed (also electronic supplementary material).

The initial value of each dependent variable was added as a covariate to avoid subtle initial biases. The initial redness values were the standardized residuals of a model that included bill brightness and surface as covariates (both p 's < 0.01). T3 and carotenoid values were log-transformed to attain normality, whereas a binomial model with logit link was used to test moult occurrence. LSD *post hoc*s were used for pairwise comparisons. Satterthwaite DFs were used. Least square means $\pm$ standard error (LSM $\pm$ s.e. hereon) from models are reported. Cohen's d effect sizes are shown for comparisons reporting $p < 0.10$.

3. Results

(a) Hormone treatment validation

The hormonal treatment factor (i.e. the four-level factor) did not show a significant effect on plasma T3 levels ($F_{3,64.1} = 1.66$, $p = 0.184$; see also electronic supplementary material). However, the plasma levels of hormone controls tended to be lower than those reported by birds receiving T3-2 and T3-3 dosages (i.e. $p = 0.073$ and 0.059 , respectively; electronic supplementary material, figure S1A). Moreover, a significant effect was found when comparing hormone controls with other birds as a single group ($F_{1,67.3} = 4.07$, $p = 0.048$; LSM $\pm$ s.e.: 6.66 ± 1.01 and 9.04 ± 0.60 ng ml⁻¹, respectively; $d = 0.50$).

The hormone treatment also increased the breath rate (hormone treatment: $F_{3,70.7} = 8.64$, $p < 0.001$). Birds from

any T3 dosage showed higher rates than hormone controls (all pairwise- p 's < 0.001 ; electronic supplementary material figure S1B in electronic supplementary material). Moult occurrence was also more frequent among hormone-treated birds, with 5, 55, 85 and 91% from controls to higher dosages ($F_{3,81} = 7.28$, $p < 0.001$; electronic supplementary material).

Birds in any hormone-treated group lost more body mass from the start of the experiment to the intermediate measure than hormone controls (hormone treatment: $F_{3,65.7} = 15.09$, $p < 0.001$; all pairwise p -values < 0.001 ; see electronic supplementary material figure S2A and electronic supplementary material, table S2). T3-3 birds also lost more body mass than T3-1 birds ($p = 0.032$). The mitoTEMPO treatment favoured body mass maintenance ($F_{1,41} = 4.38$, $p = 0.043$), but did not interact with the hormone treatment ($p = 0.30$). Furthermore, the addition of body mass change (%) as a covariate to gene expression, bill redness and carotenoid level models did not alter their tests (described in electronic supplementary material). Lastly, when testing the body mass difference from the beginning to the end of the study, only the hormone factor remained significant ($p < 0.001$), reporting similar body mass reductions that those described at the intermediate measures (electronic supplementary material).

(b) Circulating carotenoids

No treatment or interaction showed a significant effect in the model testing plasma carotenoid levels (table 1 and electronic supplementary material).

(c) CYP2J19 expression

A highly significant interaction between the antioxidant and hormonal treatments was detected (table 1). MT versus S comparisons (i.e. figure 1a) were, or tended to be, significant among hormone controls and at the two highest T3 dosages (i.e. T3-0: $p = 0.035$, $d = 0.92$; T3-2: $p = 0.032$, $d = 0.99$; T3-3: $p = 0.055$, $d = 0.85$). A global view suggests a U-shape relationship with hormone dosages among serum-only injected birds (S), whereas an inverted U appears among mitoTEMPO-injected animals (figure 1a; also electronic supplementary material). Thus, S-injected birds reported lower gene expression at the medium-sized (T3-2) dosage compared to hormone controls ($p = 0.014$, $d = 1.07$; other comparisons among S-injected birds: $p > 0.082$). In mitoTEMPO-treated birds, the control versus T3-2 reported $p = 0.066$ ($d = 0.83$), suggesting increased gene expression (figure 1a). The value then declined at the highest T3 dosage (T3-1 versus T3-3: $p = 0.027$, $d = 0.97$; T3-2 versus T3-3: $p = 0.009$, $d = 1.21$; other p -values > 0.17). It is worth noting that when circulating carotenoids at bill sampling time were added as a covariate, they were positively correlated to gene expression (slope $\pm$ s.e.: $+0.059 \pm 0.029$; $F_{1,72.5} = 4.18$, $p = 0.045$; see electronic supplementary material), but this did not modify the interaction ($p = 0.013$). Moreover, the significance of that correlation depended on two extreme values (see electronic supplementary material, and electronic supplementary material figure S4).

(d) Bill redness

The interaction between treatments (table 1) reported a trend to significance ($p = 0.064$; other terms in the model are

Table 1. Best fitted models testing the impact of the mitoTEMPO and T3 treatments on male zebra finch plasma carotenoids, bill redness and *CYP2J19* expression.

	slope $\pm$ s.e.	F	d.f.	p
circulating carotenoids				
mitoTEMPO treatment		0.21	1,42.3	0.651
T3 treatment		1.68	3,65.6	0.181
mitoTEMPO $\times$ T3 treatments		0.08	1,65.8	0.973
initial value	-0.002 ± 0.003	0.51	1,72.3	0.477
CYP2J19 expression level				
mitoTEMPO treatment		0.71	1,42.2	0.405
T3 treatment		1.20	3,65.6	0.318
mitoTEMPO $\times$ T3 treatments		4.15	3,67.5	0.009
bill redness				
measured area (mm ²)	0.058 ± 0.026	4.89	1,62.1	0.031
bill brightness	-6.476 ± 0.965	45.08	1,66.9	<0.001
initial bill redness (residuals)	0.502 ± 0.103	23.76	1,65.2	<0.001
location in the cage		7.58	1,30.9	0.010
mitoTEMPO treatment		0.01	1,36.2	0.943
T3 treatment		0.18	3,58.1	0.908
mitoTEMPO $\times$ T3 treatments		2.54	3,65.1	0.064

explained in the electronic supplementary material). That interaction would be driven by differences between antioxidant treatments (MT versus S) in the two largest T3 dosages (figure 1b). Among S-injected birds (antioxidant controls), bill redness increased from T3-2 to T3-3 dosages ($p = 0.032$, $d = 0.98$). Contrarily, among mitoTEMPO-injected birds, bill redness showed a trend to a significant decline from T3-2 to T3-3 ($p = 0.094$, $d = 0.76$; figure 1b). Lastly, mitoTEMPO-treated birds showed significantly paler bills than antioxidant controls ($p = 0.048$, $d = 0.88$) at the highest hormone dosage, thus cancelling out the trend to the positive effect of the thyroid hormone on redness.

In an alternative model, *CYP2J19* expression level was added to the table 1 model as an additional covariate (electronic supplementary material, table S4). The bill redness was positively correlated to gene expression level (covariate: $F_{1,70.6} = 5.09$, $p = 0.027$; slope $\pm$ s.e.: 0.230 ± 0.102 ; also electronic supplementary material, figure S3), whereas the interaction between both treatments became non-significant ($p = 0.189$; electronic supplementary material). The circulating levels of carotenoids did not affect the redness model (covariate always $p > 0.30$; also electronic supplementary material).

4. Discussion

Here, we show that *CYP2J19* expression levels at the bill epidermis and the expression of a red carotenoid-based trait are both affected by a mitochondrial antioxidant and also influenced by a thyroid hormone. The mitoTEMPO effect supports the involvement of mitochondria function in sexual signalling (i.e. the mitochondrial function hypothesis; [22]) and, consequently, the idea of ketocarotenoid-based ornaments evolving as quality indices [3]. By contrast, the interaction with the T3 dosages opens the theoretical

possibility of adaptive phenotypic plasticity on trait expression controlled by thyroid function (see below). This implies that these traits might have evolved as signals subjected to possible hormone-related production costs and even potential cheating ([1], but see also [6]).

The T3-treated birds reported significantly higher plasma hormone levels than controls, as well as higher breath rate, body mass loss and moult occurrence. All these T3-induced effects are well supported by avian literature [29,41,53,54] and indicate that implants were indeed effective. Moreover, plasma hormone concentrations among our T3-treated birds were within the values reported for developing zebra finches under captivity ([55], also electronic supplementary material), although nothing is still known about T3 levels in wild zebra finches. Nonetheless, plasma T3 values are surely highly variable within the same bird species due to activity, temperature, breeding cycles, age, etc. [29], and large species-related differences have also been reported (see electronic supplementary material). On the other hand, the lack of significant differences in circulating hormone levels among our specific T3 dosages could be interpreted as a result of a negative feedback regulation to avoid excessive hormone circulation at the end of the experiment [41,56,57]. The dosage-related effects detected on bill redness could, however, be a consequence of differences in plasma levels days before blood sampling.

Regarding the *CYP2J19* expression level, we should first highlight that mitoTEMPO and T3 treatments did not influence circulating carotenoid levels, and the addition of these levels as a covariate to the *CYP2J19* model, even when reporting a positive correlation (electronic supplementary material, figure S3), did not alter the results. In zebra finches, red ketocarotenoids are produced at the beak tissue from yellowish carotenoids carried in blood (i.e. lutein, zeaxanthin; [10,39] see also electronic supplementary material). Accordingly,

Figure 1. Treatment effects on the *CYP2J19* expression level (a) and bill redness (b). Hormone dosages: control, T3-1 (6 mm implant), T3-2 (8 mm) and T3-3 (12 mm). Birds injected with serum-only (S) or serum plus mitoTEMPO (MT). LSM ± s.e. from mixed models.

the *CYP2J19* gene has been detected in the red ornament tissue in this species, but not in the liver such as found in other birds that transport red carotenoids in blood [13,39]. The experimental effects thus appear to be independent of changes in substrate availability (yellow carotenoids; [39]). This independence supports the view that the reliability of carotenoid-based signals might not be explained by costs derived from resource allocation trade-offs between coloration and homeostasis maintenance [58] such as initially defended (e.g. [16,17]).

Nonetheless, even when the statistical significance of the relationship between *CYP2J19* expression and plasma carotenoid levels depended on two extreme carotenoid values (electronic supplementary material figure S4) and the plasma carotenoid level covariate did not alter the experimental effects (also electronic supplementary material), the detected covariance may still support certain dependence on resource availability to produce the signal. However, this would require future experimental approaches to manipulating dietary carotenoid availability.

In any event, mitoTEMPO and T3 treatments significantly interacted on gene expression, but the outcome did not follow our initial predictions. MitoTEMPO downregulated *CYP2J19* expression (see hormone controls in figure 1a), although this was not reflected in bill redness in that group (interpretation below). Such a ketolase downregulation contradicts the effect of another mito-targeted molecule (mitoQ) improving zebra finch bill redness [26]. This supposed incoherence could be due to differences between mitoQ and mitoTEMPO mechanisms of action. The first is synthetic ubiquinone that improves electron transfer in the IMM, whereas mitoTEMPO is a superoxide dismutase (SOD) mimetic that favours the ubiquinone

recycling by decreasing superoxide production [25,42,46]. Moreover, mitoTEMPO has a shorter alkyl chain than mitoQ. This is likely to favour its antioxidant action, avoiding mitochondrial membrane disruptions and, again, superoxide production [43–45]. Accordingly, a comparison of a very similar compound (i.e. mitoTEMPOL) with mitoQ reported lower levels of superoxide generation in human cell lines treated with the former [59]. We may thus hypothesize that mitoTEMPO induced a substantial reduction in superoxide levels (see [42]). This reduction could have interrupted cell redox signalling mechanisms linked to gene expression. Superoxide is a well-known redox signal affecting the expression of many genes (reviewed in [60,61]), and mitoTEMPO can induce downregulation of human inflammatory and cancer-related genes by interfering redox signalling pathways as a result of a decline in mitochondrial superoxide generation [62,63].

MitoTEMPO-induced *CYP2J19* downregulation would also contradict a recent study where male captive red crossbills treated with the same antioxidant and dosage produced redder feathers after experimental plucking [38]. Interestingly, however, the effect was only detected among the reddest birds at the start of that experiment (supposedly the high-quality animals; [38]). That finding suggests that other factors linked to individual quality can be constraining the hypothetical mitochondrial control on ketolase function. The mitoTEMPO × T3 interaction here detected could shed some light on this regard.

CYP2J19 downregulation induced by mitoTEMPO vanished in T3-1 and T3-2 dosages (figure 1a). By contrast, the hormone treatment inhibited *CYP2J19* expression in the same dosage range when administered alone. Thyroid hormones have been linked to high ROS generation and oxidative stress both in mammals and birds (reviewed in [33,64–66]) due to increased oxidative metabolism (i.e. oxygen consumption rate) (e.g. [67]), and we know that excessive ROS can also inhibit CYP expression and activity via negative feedback regulation (e.g. [68]). The mitoTEMPO treatment at T3-2 dosage reversed the inhibitory effect of T3 and even induced higher *CYP2J19* expression compared to hormone controls treated with the antioxidant. At the highest hormone dosage, however, the birds seem to have been able to trigger some compensatory mechanism against thyroid-mediated impairment as gene expression reaches a level that did not differ from controls (figure 1a).

Literature supports the capacity of thyroid hormones to mount compensatory/protective responses against its own pro-oxidant effects (e.g. reviewed in [69,70]). In birds, T3-treated chickens increased SOD activity compared to controls, avoiding oxidative damage [71]. In the same line, T3-treated Muscovy ducklings (*Cairina moschata*) upregulated the gene expression of uncoupling proteins (UCPs) located at the IMM [72]. These proteins are involved in decoupling cell respiration from ATP synthesis, decreasing superoxide generation [72]. Nonetheless, mitoTEMPO seems to have been able to disable that compensation at T3-3 dosage perhaps by interfering with redox signalling pathways such as suggested for the hormone control group (above). In any event, the interacting effect of the mitochondrial antioxidant and thyroid hormone on ketolase expression suggests that certain levels of ROS are needed to maintain an efficient carotenoid transformation (see also [17]).

Regarding colour expression variability, it seems to, at least partially, resemble the gene expression pattern (figure 1). However, the mitoTEMPO-induced *CYP2J19* downregulation did not lead to paler bills among the hormone controls. We should, first, consider post-transcriptional regulation mechanisms buffering the effect on the enzyme (e.g. [73]). Nonetheless, the finding could also be due to a delay in the mitoTEMPO effect on coloration. The carotenoid synthesis should not only depend on the number of *CYP2J19* copies, but on enzyme activity, and we should remember that the experiment was performed in a limited number of days. Moreover, *CYP2J19* expression and redness varied in concert at the two highest T3 dosages, which suggests that the impact of mitoTEMPO on the phenotype could be quicker as a consequence of the T3 influence on overall metabolism.

Thyroid hormone levels have been the focus of many studies addressing the evolutionary consequences of phenotypic plasticity, particularly among amphibian and fish species [27,28], but also in birds, at least during the first developing phases [74,75]. The influence of thyroid hormones on avian carotenoid-based colorations has, however, been ignored until now. The results suggest that the expression of red ketocarotenoid-based ornaments could be plastic via thyroid function control. In that case, plasticity in ketocarotenoid-based ornament expression might potentially be adaptive [76,77]. Ketocarotenoid-based colorations would not have evolved as uncheatable quality indices, such as originally proposed in the mitochondrial function hypothesis [3] but perhaps as classical condition-dependent signals [1,78]. If this is true, *CYP2J19* function could be linked to costs versus benefits balance derived from sustaining higher or lower thyroid hormone levels. For instance, a positive association exists in birds and other vertebrates [29]. Under low temperatures, high hormone levels are secreted to thermoregulate and survive. This might perhaps constrain the *CYP2J19* expression level, such as reported in T3-2 birds compared to controls (figure 1a). This would ultimately affect carotenoid transformation rates and trait expression. Those birds with better subcutaneous fat reserves or plumages (i.e. insulation) could avoid increasing thyroid hormone secretion, eluding an impairment in colour expression. This avoidable

constraint might confer honesty to the signal. Moreover, the highest T3 dosage seems to reverse gene downregulation, with redness also increasing from T3-2 to T3-3 (figure 1b). If higher T3 levels annulate the supposed constraint, physiological costs linked to a high thyroid function (e.g. oxidative damage from uncontrolled ROS or hyperthyroidism-related pathologies; [31–34,70]) could also assure signal reliability. Only those individuals able to avoid these costs could express bill redness under high thyroid levels. Anyway, unknown unavoidable constraints linked to thyroid hormones could also exist, preventing cheating. In the latter case, ketocarotenoid-based ornaments might still act as quality index signals such as discussed above.

It is evident that a full understanding of the mechanism controlling red ketocarotenoid-based signalling still requires new causal approaches involving more experiments. This should include testing mitochondrial superoxide generation and ketocarotenoid levels at the ornament tissue, and also address the proposed pathways in wild animals under free-living conditions, including the manipulation of dietary carotenoid availability. In any event, our results provide a new perspective to address the challenge.

Ethics. This experiment was approved by the Bioethics Committee of CSIC and Junta de Castilla La Mancha (JCCM) (ref number 16-2017). All the authors consent to participate.

Data accessibility. Data are open access at our Institutional public repository DIGITAL.CSIC: <http://dx.doi.org/10.20350/digitalCSIC/12664>.

Authors' contributions. C.A.A. and A.C. designed the study, carried out the experiment and analysed data; C.A.A. and A.C. led the writing of the manuscript; M.C., P.C. and A.M.B. were involved in laboratory analyses and manuscript reviewing.

Competing interests. We declare we have no competing interests.

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