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ORIGINAL ARTICLE

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Carotenoid-based plumage coloration is not affected by vitamin E supplementation in male greenfinches

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Abstract Carotenoid-based colours have become an important model of honest signalling as carotenoids are suggested to play vital roles in several physiological functions including antioxidants and immunostimulators, while they are also required for sexual displays. However, it has been recently suggested that carotenoid-based signals may be used mainly as reflectors of the systems that prevent their oxidation (mainly the amount of other non-pigmented antioxidants) rather than the antioxidative properties of carotenoids themselves. We tested this hypothesis by examining the effect of simultaneous supplementation of carotenoids and an uncoloured antioxidant—vitamin E—on the coloration of growing tail feathers in captive male greenfinches (*Carduelis chloris chloris* L.). While carotenoid supplementation enhanced the coloration of the feathers, manipulation of dietary vitamin E had no effect. Thus, our results do not support the idea that carotenoids are mainly used as indicators of the abundance of other antioxidants.

Keywords *Carduelis chloris* · Dietary antioxidants · Carotenoid supplementation

Introduction

Carotenoid-based signals have been the target of extensive research by animal ecologists since the recognition of their possible role in health maintenance and signalling. As the carotenoids must be acquired from food (Fox 1979) and are destroyed when used as antioxidants (Vershinin 1999), it is believed that their use as colorants in ornaments, such as feathers, skin and scales, may be traded off with their use in other functions (e.g. as immunostimulators or antioxidants). Therefore, it has been suggested that carotenoid-based visual characters enable individuals to convey honest information on their bearer's phenotypic and/or genetic quality to potential mates and opponents (e.g. Lozano 1994; Olson and Owens 1998; Møller et al. 2000; Hill and McGraw 2006). Only the highest quality individuals could allocate sufficient quantities of pigments to develop the most colorful signals without compromising the essential need of carotenoids for maintenance purposes at the same time.

In birds, the idea that carotenoid-based signals function as badges of individual quality has received considerable support from correlational and experimental studies that link the colour of such traits to foraging efficiency, disease status and immunocompetence (review in Hill and McGraw 2006). In this context the antioxidative nature of carotenoids is highly advocated (Lozano 1994; Olson and Owens 1998; von Schantz et al. 1999; Møller et al. 2000). Still, with few exceptions (Jaensch et al. 2001; Woodall et al. 1996), the antioxidant function of carotenoids in birds has been studied mainly in the context of embryo-protective maternal effects (Surai 2002; McGraw et al. 2005). Only a few studies (Alonso-Alvarez et al. 2004; Bertrand et al. 2006a; Costantini et al. 2006) have considered the relationships between carotenoids and general antioxidant defences in postembryonic stage. Furthermore, the idea that carotenoid-based ornaments are direct displays of their bearers antioxidative power via carotenoid compounds has been recently challenged by Hartley and Kennedy (2004). They argue that although carotenoids do exhibit antioxidant activity in vitro, this is not their primary biological role. Instead, they suggest that carotenoids may be used mainly as signals, revealing the amount of non-pigmented antioxidants (such as antioxidative enzymes and vitamins E and C), which are more important biological protectants against free-radical-mediated oxidative stress. Under this scenario, organisms rely on carotenoids to advertise their antioxidative potential as the presence of other (uncoloured) antioxidants would protect carotenoids from oxidative

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decoloration. To our knowledge, this hypothesis of Hartley and Kennedy (2004), that carotenoid-based coloration is affected by the availability of other antioxidants, has been tested only twice (Bertrand et al. 2006b; Pike et al. 2007). Supplementation with uncoloured antioxidants (melatonin and mixture of vitamins E and C, respectively) had an additive effect on the carotenoid-based ornamental coloration of the beaks in captive zebra finches (*Taeniopygia guttata*; Bertrand et al. 2006b) and nuptial skin patches in sticklebacks (*Gasterosteus aculeatus*; Pike et al. 2007). Still, whether these results can be generalised to other antioxidants and ornaments (such as feathers) remains untested. Feather ornaments are interesting in the context of signalling because once the carotenoids are deposited in such a metabolically inactive tissue, they become unavailable for other biological functions (Olson and Owens 1998). Hence, one might expect the carotenoid-based feather ornaments to be a particularly honest and long-term signal of individual ability to cope with oxidative stress.

We tested the hypothesis of Hartley and Kennedy by experimentally supplementing captive male greenfinches (*Carduelis chloris chloris* L.) with carotenoids and an uncoloured antioxidant, vitamin E. Greenfinches express several carotenoid-based plumage patches which are sexually selected (Eley 1991). The yellow colour of these feathers is based on canary xanthophylls A and B and their *cis* isomers (Saks et al. 2003), which are produced by interconversion of dietary lutein and zeaxanthin (Surai 2002). Intensity of the yellow coloration of male greenfinches reflects the amount of carotenoids deposited into feathers (Saks et al. 2003) and plasma carotenoid levels during feather growth (Karu et al. 2007). Carotenoid-based plumage coloration of male greenfinches is sensitive to intestinal (Hõrak et al. 2004b), viral (Lindström and Lundström 2000) and hematozoan (Merilä et al. 1999) infections.

Vitamin E is the main lipophilic antioxidant in animals, and it can only be procured from food. Due to the ability to scavenge peroxyl radicals and singlet oxygen, it is probably the most important agent in cell membrane defences as an inhibitor of the free-radical chain reaction of lipid peroxidation (Halliwell and Gutteridge 2004). Thus, the abundance of vitamin E can be expected to significantly affect the total antioxidative potential of an individual organism. Therefore, if the primary role of carotenoid-based signals is to reflect the levels of other antioxidants, then supplementary feeding with vitamin E should have a significant effect on the expression of carotenoid pigmented ornaments. Thus, we predicted that simultaneous supplementation of vitamin E and carotenoids would result in more colourful feathers than carotenoid supplementation alone.

Materials and methods

Ninety-four male greenfinches were caught in mist-nets in the Sõrve Bird Observatory on Saaremaa island (57°55′N; 22°03′E) between 20 (day 1 of the experiment) and 22 January 2006. Birds were transported to Tartu a day after capture and housed in individual indoor cages (27 × 51 × 55 cm) with sand bedding. Ad libitum sunflower seeds and filtered tap water were always available. Sunflower seeds contain on average 720 μg/g of tocopherols (Yoshida et al. 2002) and 1 μg/g of carotenoids (McGraw et al. 2001). During the experiment, the average temperature in the aviary was 16.0 ± 1.6 (SD)°C and the average humidity was 51.5 ± 2.4 (SD)%. Natural day-length cycle was maintained in the aviary throughout the experiment. Birds were released to their natural habitat on 1 April 2006 (day 72).

After transportation to Tartu, the birds were allowed a 17-day acclimation period (days 3–19). The birds were divided into six treatment groups (15–16 birds in each). These groups were set to have similar average body mass (group averages ranging from 28.1 to 28.8 g ± 1.7–2.0 SD) and age composition (8–9 first year and 7 older birds in each group). In the morning of day 20, the birds in each treatment group started to receive different antioxidant supplementations. Carotenoid dosage was based on our previous experience showing that supplementation with 10 μg/ml carotenoid solution during 33 days resulted in significant increase in plasma carotenoid levels (Hõrak et al. 2006) and lab-grown feather chroma (Karu et al. 2007) in supplemented birds as compared to controls. In this experiment, we increased the concentration of the solution (12 μg/ml), as the supplementation time was planned shorter (13 days). High dose of vitamin E was set to approximately double the content of tocopherols (mainly α-tocopherol) contained in sunflower seeds (Yoshida et al. 2002); low dose of vitamin E was 50% of high dose. The treatments were formed as follows (Fig. 1): (1) Carotenoid—birds received 12 μg/ml carotenoid solution on alternate days, altogether in 13 days. (2) High vitamin E—birds received 500 μg/ml tocopherol solution on alternate days, altogether in 13 days. (3) Low vitamin E—birds received 250 μg/ml tocopherol solution on alternate days, altogether in 13 days. (4) Carotenoid + high vitamin E—birds received 12 μg/ml carotenoid solution and 500 μg/ml tocopherol solution on alternate days, altogether 26 days of antioxidant supplementation. (5) Carotenoid + low vitamin E—birds received 12 μg/ml carotenoid solution and 250 μg/ml tocopherol solution on alternate days, altogether 26 days of antioxidant supplementation. (6) Control—birds received filtered tap water only. Carotenoid supplementation consisted of lutein and zeaxanthin (20:1, *w*/*w*), prepared from OroGlo liquid solution of 11 g/kg xanthophyll activity (Kemin AgriFoods Europe, Herentals, Belgium). Vitamin E supplementation was prepared from water-soluble vitamin E solution Aqua-E™ (Yasoo Health Inc., Johnson City, TN) which consisted of a mixture of 20 mg/ml *d*-α-tocopherol, 12 mg/ml *d*-γ-tocopherol, 4 mg/ml *d*-β-tocopherol + *d*-δ-tocopherol (respectively 56, 33 and 11% from total tocopherols) and 2 mg/ml total tocotrienols. In sunflower seeds, α-tocopherol is

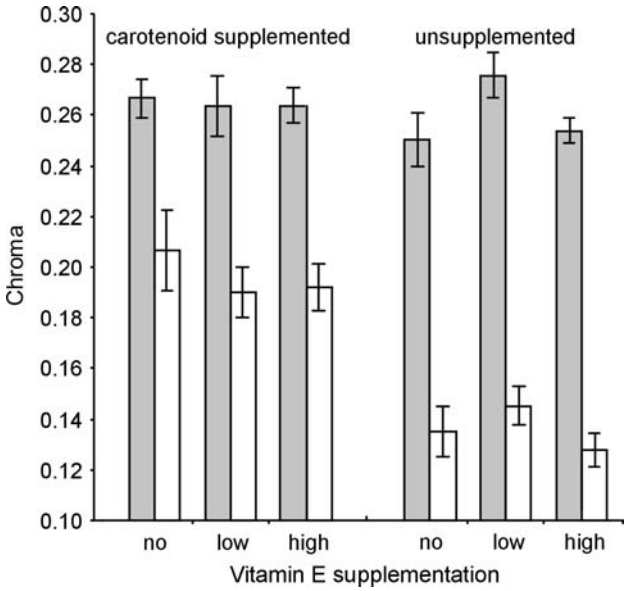

Fig. 1 Average (±SE) chroma values of wild-grown (*filled columns*) and replacement feathers grown during the experiment (*empty columns*) of different treatment groups (n = 15 in all groups)

predominant (over 90%), while γ- and β-tocopherol account for 6 and 1%, respectively (Yoshida et al. 2002). Similarily, in greenfinches, kept on sunflower seed diet, α-tocopherol formed 95% and γ-tocopherol 5% of total measured tocopherols (Hõrak et al. unpublished data). Supplement solutions were freshly prepared each evening using filtered (Brita® Classic; BRITA GmbH, Taunusstein, Germany) tap water and were provided in 50-ml doses in opaque dispensers in order to avoid oxidation of carotenoids and vitamin E. The effect of these supplementations on individual measures of antioxidants (carotenoids and uric acid), plasma antioxidant potential and lipid peroxidation products are discussed in Hõrak et al. 2007. We were unable to measure vitamin E hemoconcentrations due to technical reasons.

For the analysis of plumage colour, one right outermost (sixth) tail feather (wild-grown feathers) was collected from each individual during the 20th day of the experiment. Full-grown replacement feathers (lab-grown feathers) were collected from the same positions right before the birds were released (on the day 71). Collected feathers were stored in plastic bags in the dark. Colour measurements were performed as described in detail by Karu et al. (2007) and Hõrak et al. (2004b) using a spectrophotometer (Ocean Optics USB2000 with Ocean Optics DH2000 lamp). Plumage colour was characterised on the basis of chroma (see Endler 1990 for details) of the yellow parts of tail feathers. Measuring error of this method of plumage colour quantification is reasonably low as the repeatability (Lessells and Boag 1987) of three consecutive measurements of the same feather ranged from 0.73 to 0.88 (Saks et al. 2003).

Table 1 The effect of supplementary vitamin E and carotenoid feeding on feather chroma in repeated measures ANOVA

Effect	*df*	*F* value	*P* value
Univariate tests			
Carot.	1;84	33.96	< 0.00001
Vit. E	2;84	0.94	0.39
Carot. × Vit. E	2;84	2.23	0.11
Multivariate tests			
Time	1;84	305.45	< 0.00001
Time × Carot.	1;84	25.39	< 0.00001
Time × Vit. E	2;84	0.64	0.53
Time × Carot. × Vit. E	2;84	0.006	0.99

"Carot." and "Vit. E" stand for carotenoid supplementation and vitamin-E supplementation, respectively. "Time" represents the difference in chroma of wild-grown versus lab-grown feathers within individuals

The effect of the treatments on the feather chroma were examined with repeated-measures ANOVA, assuming that the effect of treatment would be revealed by significant "time × treatment" interaction term, where "time" denotes within-individual differences in wild-grown and lab-grown feathers. All reported *P* values are calculated for two-sided tests.

Results

Carotenoid treatment had a significant effect on feather chroma (Table 1; Fig. 1). Lab-grown feathers of carotenoid supplemented birds had significantly (30.6%) higher chroma as compared to the feathers of unsupplemented individuals. Vitamin E supplementation did not affect the change in feather colour.

Discussion

The manipulation of dietary vitamin E availability did not affect the coloration of lab-grown feathers although simultaneous carotenoid supplementation had significant positive effect on the chroma values of these ornaments. These findings do not support the hypothesis, put forward by Hartley and Kennedy (2004), that carotenoid-based ornaments serve mainly as indicators of the abundance of other (uncoloured) antioxidants. Our results also differ from previous findings demonstrating that supplementary feeding with uncoloured antioxidants—melatonin (Bertrand et al. 2006b) and vitamins E and C (Pike et al. 2007) resulted in enhancement of carotenoid-based coloration in captive male zebra finches and sticklebacks, respectively. These discrepancies can be explained either by the different antioxidants, experimental methodology or by different ornaments and species studied.

We acknowledge that also some confounding factors, rather than the absence of biologically meaningful relationships, may be responsible for the lack of effect of vitamin E supplementation on feather coloration. For

instance, it could be argued that the supplemented micellized tocopherols were not at all, or were not easily absorbable for the birds. However, this is unlikely, as it has been shown that micellized vitamin E supplements effectively increase circulating plasma tocopherol levels (Ochoa et al. 1992; Traber et al. 1994).

Another, more likely confounding factor might be that high tocopherol concentration of the base diet masked the effect of vitamin E supplementation. Sunflower seeds are rich in vitamin E (Yoshida et al. 2002), while being a relatively poor source of carotenoids (McGraw et al. 2001). It has been suggested that if α-tocopherol is ingested in large doses, much is not absorbed and is excreted (Halliwell and Gutteridge 2004). Higher doses of vitamin E produce relatively small increases in plasma tocopherol levels in humans (Burton et al. 1998) and barn swallows (*Hirundo rustica*; de Ayala et al. 2006). However, at present it is largely unknown whether and how limiting nutrient vitamin E is in wild birds. Most of the studies reporting the beneficial effects of vitamin E supplementation in birds have concentrated on domestic species under strict artificial diets, or embryos and juveniles, which exhibit extremely high metabolic rates and may experience extensive oxidative stress (e.g. Surai 2002). A single study of wild passerines (de Ayala et al. 2006) found that relatively low physiological doses had small and transient effects on nestling growth and condition in barn swallows, whereas a higher physiological dose did not enhance offspring quality. Plasma α-tocopherol levels of captive greenfinches on sunflower seed diet (20.6–26.8 μg/ml; Hõrak et al. 2004b) are relatively high compared to the concentrations reported in several other wild passerines (< 13 μg/ml; e.g. Biard et al. 2005; de Ayala et al. 2006; Ewen et al. 2006; but see Hõrak et al. 2004a). Thus, we cannot totally exclude the possibility that the effect of vitamin E supplementation in our study had no effect on feather coloration because all birds received already the maximum absorbable concentration of tocopherols from their sunflower seed diet.

We found that while lab-grown feathers were all duller than wild-grown feathers, dietary carotenoid supplementation resulted in increased feather chroma compared to unsupplemented individuals. This result is not surprising and compares favourably with many previous studies (reviewed in Hill and McGraw 2006). The ''bleaching'' of the feather coloration in captivity has been reported previously (discussed in detail e.g. in Hõrak et al. 2004b; Hill and McGraw 2006) and can be most likely ascribed to insufficient carotenoids (or other micronutrients required for carotenoid biotransformations, transportation and deposition to integument) in the diet. The significant effect of dietary carotenoid supplementation has been reported in many different avian species (reviewed in Hill and McGraw 2006). Altogether, the result that increased carotenoid availability is mirrored in sexual ornaments is consistent with the current understanding of the mechanics of carotenoid-based signalling (e.g. Møller et al. 2000; Surai 2002).

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