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Do carotenoid-based sexual traits signal the availability of non-pigmentary antioxidants?

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Summary

Carotenoid-based signals are thought to be indicators of male quality because they must be obtained from the diet and might thus indicate the ability of individuals to gather high-quality food. However, carotenoids are also known to have important physiological functions as immunoenhancers and antioxidants, and, as such, carotenoid-based sexual traits have also been suggested to reflect the health and antioxidant status of their bearers. This last idea is based on the hypothesis that carotenoids that are allocated to sexual signals are no longer available for the detoxification system. Recently, this hypothesis has been challenged on the grounds that the antioxidant activity is not the main biological role of carotenoids. Instead, carotenoid-based sexual traits might signal the availability of other non-pigmentary antioxidant molecules that might protect carotenoids from free radical attacks and make them available for sexual advertisements. We tested this hypothesis in the zebra finch, a passerine species with a carotenoid-based signal: the colour of the bill. We simultaneously manipulated the availability of carotenoids and of a non-pigmentary antioxidant (melatonin) in the drinking water. If the antioxidant properties of melatonin protect carotenoids from oxidation, we predict that birds supplemented with melatonin should have redder bills than birds not supplemented with melatonin, and that birds supplemented with carotenoids and melatonin should have redder bills than birds supplemented with carotenoids alone. Our findings are in agreement with these predictions since carotenoid and melatonin supplementation had an additive effect on bill colour. To our knowledge this is the first experimental evidence that a non-pigmentary antioxidant enhances the expression of a carotenoid-based sexual trait.

Key words: carotenoids, free radicals, melatonin, oxidation, sexual advertisement, zebra finch.

Introduction

Many animals, such as fish and birds, use carotenoid-based signals during the process of sexual selection (Olson and Owens, 1998), in particular during mate choice. The honesty of carotenoid-based signals is thought to be reinforced by two non-exclusive mechanisms. First, since carotenoids are ultimately produced by plants and must be absorbed by animals from their diet to produce sexual signals (Kodric-Brown, 1985; Kodric-Brown, 1989; Hill, 1991; Badayev and Hill, 2000), the capacity to produce carotenoid-based coloration would be ultimately linked to the individual ability to acquire, assimilate, and process carotenoids (Endler, 1980; Hill, 1990; Hill, 1999). Second, because carotenoids have important physiological functions as immunoenhancers (Lozano, 1994; Chew and Park, 2004) and free radical scavengers (von Schantz et al., 1999; Krinsky, 2001), only high quality males could afford the cost of using carotenoids in sexual signals. This hypothesis is based on the assumption that carotenoids are limiting, and that carotenoids allocated to the signal are no longer available as immunoenhancers or antioxidants. The role of carotenoids as modulators of the immune response has recently received much attention (Chew and Park, 2004). In a few studies, carotenoid supplementation has been shown to improve both the expression of sexual signals and the immune response (Blount et al., 2003; McGraw and Ardia, 2003), presumably because of a direct effect of these pigments on tegumentary colour and immune functioning. Similarly, an experimental activation of the immune system has been shown to reduce the expression of carotenoid-based signals and to reduce the amount of carotenoids in the plasma of two bird species (Faivre et al., 2003; Alonso-Alvarez et al., 2004) (see also Navara and Hill, 2003).

The other potential important function of carotenoids is as antioxidants (Krinsky, 2001; El-Agamey et al., 2004). The antioxidant action of carotenoids occurs through the scavenging of free-radicals (Burton and Ingold, 1984;

Mortensen and Skibsted, 1996) and the quenching of singlet oxygen (Conn et al., 1991; DiMascio et al., 1989). Moreover, epidemiological studies have shown that dietary carotenoids can be associated with protection from certain age-associated diseases, such as cancer, cardiovascular and inflammatory diseases (Schabath et al., 2004; Tamimi et al., 2005; Walston et al., 2005). Other studies have, however, failed to find such an association and, therefore, we still do not have a complete picture of the importance of carotenoids as antioxidants *in vivo* (El-Agamey et al., 2004; Hartley and Kennedy, 2004). In addition, carotenoids can even have a pro-oxidant activity (Burton and Ingold, 1984) at high doses (higher than the amount normally ingested).

Based on the findings that carotenoids do not always have a protective role against free radicals, and that oxidation alters or destroys their colour (thus reducing their sexual signalling function), it was suggested that carotenoid-based sexual traits might signal the abundance of the non-pigmentary antioxidant molecules that protect carotenoids from oxidation and make them accessible for sexual signalling (Hartley and Kennedy, 2004). A complex antioxidant machinery is indeed available to scavenge free radicals. These antioxidant defences include enzymatic (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) as well as non-enzymatic free radical scavengers (vitamins C and E, uric acid, melatonin, cysteine, glutathione), and minerals (selenium, zinc) (Prior and Cao, 1999; Fang et al., 2002).

The hypothesis that carotenoid-based sexual traits signal the abundance of non-pigmentary antioxidants provides testable predictions. In particular, it is straightforward to predict that by increasing the availability of non-pigmentary antioxidants we should enhance the expression of carotenoid-based sexual traits.

We tested the prediction of Hartley and Kennedy in zebra finches (Hartley and Kennedy, 2004). Male zebra finches have red bills that have been shown to be the targets of female choice (Burley and Coppersmith, 1987; Blount et al., 2003) (see also Collins and ten Cate, 1996). Moreover, the colour of the bill depends on the availability of carotenoids in the environment (Blount et al., 2003; Alonso-Alvarez et al., 2004; Bertrand et al., 2006) and immune activation reduces the availability of carotenoids for the sexual signal (Alonso-Alvarez et al., 2004). We manipulated the availability of carotenoids and of a non-pigmentary antioxidant, melatonin, in the drinking water. Melatonin is a secretory product of the pineal gland, and other organs, which has multiple functions (Hardeland and Pandi-Perumal, 2005). Among its functions, melatonin has been shown to be both a direct free radical scavenger (Tan et al., 2002) and also to stimulate a number of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GRx) (Reiter et al., 2000; Anisimov, 2003; Rodriguez et al., 2004). If the antioxidant properties of melatonin protect carotenoids from oxidation, we predict that birds given melatonin should have redder bills than birds not given melatonin, and that birds given supplements of carotenoids and melatonin should have redder bills than birds given carotenoids alone.

Materials and methods

General procedures

The study was carried out on male zebra finches *Taeniopygia guttata* Vieillot (*N*=40) housed in an indoor aviary. They were held in wire cages (0.6×0.4×0.4 m) for all 30 days of the experiment under constant temperature (21±1°C) and controlled daily light cycles (13 h:11 h L:D). All individuals were provided with food (commercial seed mix for exotic birds) *ad libitum*. Two males were kept per cage with a separation in the middle to ensure that each bird was isolated.

Birds were randomly assigned to one of four treatments (*N*=10 males per group): carotenoid supplementation in the drinking water [100 μg ml^{-1}; Oro Glo^{TM} liquid, 11 mg ml^{-1} lutein and zeaxanthin (20:1, w/w); Kemin France SRL, Nantes, France (Alonso-Alvarez et al., 2004)], melatonin supplementation in the drinking water [50 μg ml^{-1} (Moore and Siopes, 2000; Moore and Siopes, 2002)], carotenoid and melatonin supplementation in the drinking water (100 μg ml^{-1} and 50 μg ml^{-1}, respectively), control group receiving tapwater. All drinks were prepared freshly each day using cool water, provided in opaque dispensers, at the same hour of the day (13:00 h).

Body mass (±0.1 g), the amount of carotenoids in plasma, and bill colour were measured twice for each bird during the experiment (day 0 and day 30). Initial values of body mass, amount of carotenoids in plasma and bill colour did not differ between supplementation groups (all measurements, $P>0.2$).

Measuring plasma carotenoids

To assess the amount of carotenoids in the plasma, blood was collected from the brachial vein into heparinized microcapillary tubes (~150 μl). The blood was centrifuged, and plasma stored at –20°C for later measurements of total carotenoid concentration.

Colorimetry was used to determine the amount of plasma carotenoids. Plasma (20 μl) was diluted in 180 μl of absolute ethanol. The dilution was mixed in a vortex and the flocculent protein precipitated by centrifuging the sample at 1500 g for 10 min. We examined the supernatant in a spectrophotometer and determined the optical density of the carotenoid peak at 450 nm. Carotenoid concentration was determined from a standard curve of lutein. We have previously shown that spectrometric and HPLC measurements of plasma carotenoids are highly correlated in zebra finches (Alonso-Alvarez et al., 2004).

Measuring bill colour

Bill colour was assessed using a Dulux Trade Colour chart (Dulux, Asnières, France) under the same light conditions. The following specific scale, ranging from less red to redder colours, was used: 1 (69YR 34/780), 2 (56YR 28/778), 3 (44YR 26/756), 4 (34YR 20/708), 5 (31YR 18/648) 6 (16YR 16/594), 7 (19YR 13/558), 8 (09YR 11/475), 9 (14YR 10/434), where the first number and letters indicate the hue, the numerator is the brightness, and the denominator is the saturation (Blount et al., 2003). Measurements of bill colour

were always performed by the same person (S.B.) blind with respect to the treatments. We have previously shown that bill colour scores and hue values provided by image analysis software (LUCIA G 4.81 Finale Software, Nikon, Paris, France) are highly correlated in zebra finches (r=–0.867, P<0.0001, N=15), and that colour scores are highly repeatable both between and within observers (intraclass correlation coefficient: R=0.96, N=39, P<0.0001, R=0.93, N=64, P<0.0001, respectively) (P. Gautier, M. Barroca, S. Bertrand, C. Eraud, M. Gaillard, M. Hamman, S. Motreuil, G. Sorci and B. Faivre, manuscript submitted for publication).

Statistical analyses

The effect of carotenoid and melatonin supplementation on body mass and plasma carotenoids was assessed using two-way ANOVA [PROC GLM (SAS Institute, 2001)]. Both variables were $\log_{10}$-transformed to ensure the normality of the residuals (Shapiro–Wilk test, P>0.05). ANOVAs were performed on differences (post-experimental – pre-experimental values). To assess the effect of treatments on bill colour we used an ordinal model for multinomial data with a cumulative logit link function [PROC GENMOD (SAS Institute, 2001)]. As for body mass and plasma carotenoids, we computed the difference in post-experimental and pre-experimental bill colour index and used it as the dependent variable in the model.

Results

Individuals in the four experimental groups maintained similar body masses during the course of the experiment, with the exception of males that received water supplemented with both carotenoids and melatonin. These birds gained 11% of their initial body mass in the 30-day experiment (Fig. 1). This indicated a significant interaction between carotenoid and melatonin supplementation on body mass (Table 1).

As expected, plasma carotenoids significantly increased in birds that received carotenoid-supplemented water, whereas the melatonin supplementation had no effect on the amount of circulating carotenoids (Fig. 2, Table 2).

Birds that received carotenoid-supplemented water had redder bills at the end of the experiment (Table 3, Fig. 3). Interestingly, melatonin supplementation also affected bill colour, with melatonin-supplemented birds having redder bills at the end of the experiment (Table 3, Fig. 3). The interaction between carotenoid and melatonin supplementation was not statistically significant (Table 3), showing that the two treatments had an additive effect on the expression of the sexual signal.

Discussion

We found that increased availability of a non-pigmentary antioxidant enhanced the expression of a carotenoid-based sexual trait, but had no effect on the amount of circulating carotenoids. Interestingly, melatonin and carotenoid supplementation had an additive effect on the expression of bill colour, as shown by the non-significant interaction between the two treatments. Plasma carotenoids were only affected by carotenoid supplementation, with treated birds having more circulating carotenoids than untreated birds.

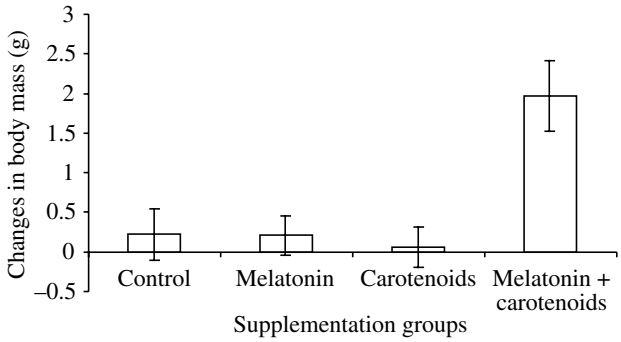

Fig. 1. Changes in body mass (g) for male zebra finches of different supplementation groups. Values are means ± s.e.m. (N=40). Changes were computed as the differences between the final and initial body masses for each group.

The role of carotenoids as antioxidants has received much attention in recent years (for example, see Krinsky, 2001). Behavioural ecologists interested in the signalling function of coloured secondary sexual traits took advantage of the supposedly antioxidant function of carotenoids to suggest that carotenoid-based signals might be used by females to assess the overall quality of mates (von Schantz et al., 1999). Recently, a modified version of this hypothesis was proposed (Hartley and Kennedy, 2004). According to this version, carotenoid-based sexual traits would indicate the antioxidant status of their bearers not because carotenoids participate in the antioxidant machinery, but because they can only be used for the sexual signal in their unbleached form. Carotenoids are very sensitive to oxidation (Woodall et al., 1997), which alters and destroys their colour. Therefore, their signalling function can only be ensured if they are protected from the oxidation. Hartley and Kennedy suggested that only individuals that have a very effective antioxidant machinery (vitamins C and E, catalase, superoxide dismutase) would have enough unbleached carotenoids available for the sexual signal (Hartley and Kennedy, 2004). Therefore, although carotenoid-based signals might still indicate the overall antioxidant status of their bearer, the mechanisms would be slightly different from the one

Table 1. *Effect of carotenoid and melatonin supplementation on body mass of zebra finches*

Sources of variation	d.f.	F	P
Carotenoid supplementation	1	5.35	0.0265
Melatonin supplementation	1	9.39	0.0041
Carotenoid + melatonin supplementation	1	8.68	0.0056
Error	36		

Differences in body mass (g; post-experimental minus pre-experimental values) were analysed using a two-way ANOVA.

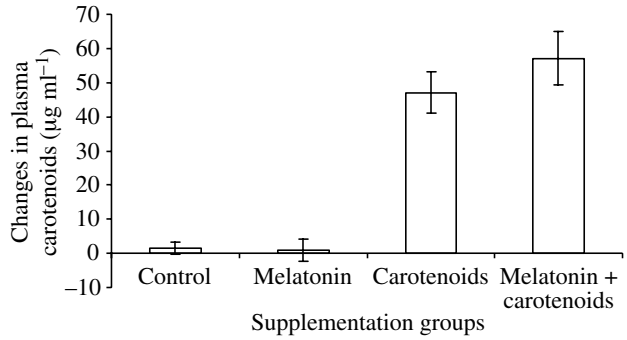

Fig. 2. Changes in plasma carotenoids (μg ml^{-1}) in male zebra finches of different supplementation groups. Values are means ± s.e.m. (*N*=40). Changes were computed as the differences between the final and the initial values of plasma carotenoids for each group.

originally envisaged. Our results are in agreement with the hypothesis of Hartley and Kennedy that antioxidant defences can prevent the bleaching of carotenoids and allow the organism to allocate them to the production of sexual signals (Hartley and Kennedy, 2004), since supplementation with melatonin, a potent non-pigmentary antioxidant, affected the expression of bill colour in male zebra finches.

Although the results reported in this study are in agreement with the hypothesis of Hartley and Kennedy (Hartley and Kennedy, 2004), we cannot completely discard another explanation. By providing birds with melatonin, we might have increased the overall availability of antioxidant molecules, and individuals might have invested more carotenoids in the sexual trait. According to this scenario, we might have made carotenoids 'less needed' for the antioxidant system. The experimental design used in this study does not allow us to tease apart the two explanations (the 'protection' and the 'carotenoid less needed' hypotheses). However, we believe that the finding that the amount of circulating carotenoids was not affected by the melatonin supplementation is supportive of the 'protection' hypothesis. Indeed, if melatonin supplementation increased the pool of antioxidants available for the organism, we might have expected the amount of circulating carotenoids to increase. Nevertheless, further work is clearly needed to tease apart the two hypotheses, as well as to identify the rules

Table 2. *Effect of carotenoid and melatonin supplementation on plasma carotenoids of zebra finches*

Sources of variation	d.f.	*F*	*P*
Carotenoid supplementation	1	86.63	<0.0001
Melatonin supplementation	1	0.21	0.6530
Carotenoid + melatonin supplementation	1	0.00	0.9809
Error	36		

Differences in plasma carotenoids (μg ml^{-1}; post-experimental minus pre-experimental values) were analysed using a two-way ANOVA.

Table 3. *Effect of carotenoid and melatonin supplementation on bill colour score*

Sources of variation	d.f.	χ^2	*P*
Carotenoid supplementation	1	19.92	<0.0001
Melatonin supplementation	1	4.87	0.0273
Carotenoid + melatonin supplementation	1	0.36	0.5501

Differences in bill colour scores (post-experimental minus pre-experimental scores) were analysed using an ordinal model for multinomial data with a cumulative logit link function [PROC GENMOD (SAS Institute, 2001)].

that govern the oxidation of carotenoids and their allocation to secondary sexual traits.

In spite of the considerable attention that has been devoted to the information content of carotenoid-based sexual traits (Olson and Owens, 1998), we still have relatively few empirical data in support of the hypothesis that individuals trade carotenoids against antioxidant protection or immune functioning. Most of this evidence comes from studies on birds and fish (Alonso Alvarez et al., 2004; Blount et al., 2003; Faivre et al., 2003; Grether et al., 2004). In zebra finches, an experiment in which birds were given variable doses of carotenoids in the drinking water showed that bill colour became redder as carotenoid availability increased until a certain dose, beyond which a further increase in carotenoid availability did not produce any change in bill colour (Alonso Alvarez et al., 2004). In the same study, activation of the immune system by means of lipopolysaccharide injections resulted in depressed bill colour and decreased amount of plasma carotenoids, whatever the availability of carotenoids in the environment. Finally, plasma carotenoids were positively correlated with the antioxidant status in these birds suggesting that there might be a trade-off between the amount of carotenoids that are allocated to the expression of a secondary sexual trait, immune functioning and the antioxidant machinery (Alonso Alvarez et al., 2004). Unfortunately, as non-pigmentary antioxidants were neither manipulated nor assessed in this study, one cannot exclude that the observed results are due to the

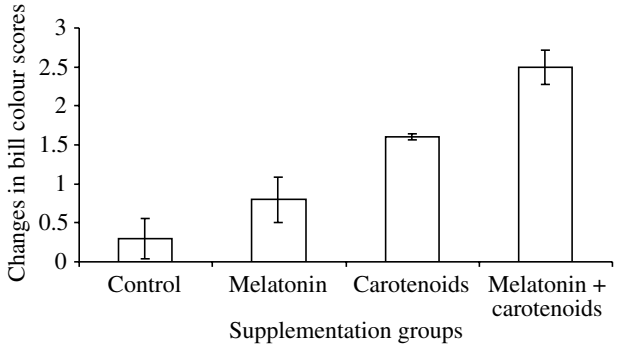

Fig. 3. Changes in bill colour scores of male zebra finches of different supplementation groups. Values are means ± s.e.m. (*N*=40). Changes were computed as the differences between the final and the initial values of bill colour scores for each group.

protection of carotenoids by vitamins or antioxidant enzymes. These results undoubtedly call for more experimental work in order to have a clearer picture on the trade-off between carotenoids allocation to sexual traits and the antioxidant function.

In this study we chose melatonin as a supplement for the birds, instead of other antioxidants, for three main reasons. First, melatonin has been reported to be a very powerful antioxidant even when compared to other free radical scavengers. *In vitro*, melatonin scavenges hydroxyl radicals, peroxides, singlet oxygen and nitric oxide (Reiter et al., 2001; Tan et al., 2002), and it has been shown that melatonin is superior to vitamin E as a peroxyl radical scavenger (Pieri et al., 1994). Compared with other major antioxidants (ascorbate and vitamin E), melatonin is also 60- and 70-fold more effective, respectively, in reducing oxidative DNA damage (assessed as the production of 8-hydroxy-2′-deoxyguanosine) (Qi et al., 2000). Second, melatonin has an indirect effect on the antioxidant machinery by stimulating antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, glutathione reductase, catalase, and other antioxidant molecules, such as ascorbate, trolox and NADH (Gitto et al., 2001), but also by inhibiting pro-oxidant enzymes such as nitric oxide synthase and lipoxygenase (Reiter et al., 2000; Anisimov, 2003; Rodriguez et al., 2004). Moreover, melatonin can also directly affect the production of reactive oxygen species by reducing electron leakage through the mitochondrial membrane (Reiter et al., 2001).

Although, in the light of these previous findings, melatonin acts as a powerful antioxidant, this hormone also intervenes in many other regulatory functions of the organism (Anisimov, 2003). Because of these multiple functions, we cannot completely discard the possibility that the observed effect on the expression of the secondary sexual trait was due to something unrelated to the antioxidant properties of melatonin. We will briefly discuss here these possible alternatives.

Melatonin has a well known function in the regulation of the circadian and seasonal rhythm (Cassone, 1990; Gwinner et al., 1997). As such, it could be that birds given melatonin might have been stimulated to enhance their secondary sexual trait as a response to a seasonal signal. However, unlike mammals, birds do not seem to use melatonin as a signal to tune their reproduction to a particularly favourable time of the year (Storey and Nicholls, 1978; Chakraborty, 1995). Moreover, in many studies on birds, reptiles, amphibians and fish melatonin administration usually suppress reproductive parameters (Mayer et al., 1997), such as testis and ovary development and spermatogenesis.

Melatonin might also have an effect on glucose and lipid metabolism. However, the results on this issue are rather controversial (Bizot-Espiard et al., 1998; Rasmussen et al., 1999; Wolden-Hanson et al., 2000; Fabis et al., 2002; Mustonen et al., 2002; Picinato et al., 2002), which makes it difficult to have a clear-cut prediction on the expected direction of the effect. We found that the body mass of birds given supplements of both melatonin and carotenoids increased substantially during the course of the experiment, compared with the birds in the other three experimental groups whose body mass remained constant. Although this result might suggest that indeed, melatonin affected either food intake, or metabolic activity of birds, it is unclear why this effect was restricted to the group receiving both melatonin and carotenoids. If melatonin had an effect on glucose and/or lipid metabolism, we should also have found a difference in body mass of birds given only melatonin.

Finally, melatonin has a regulatory effect on the immune response (Srinivasan et al., 2005). Melatonin increases the production of antibodies against T-dependent antigens, activates Natural Killer (NK) cells and monocytes, and the release of cytokines (IL-1, IL-2, IL-6, IL-12, and IFNγ) (Hardeland et al., 2006). Given that carotenoids also have an immunostimulatory effect, one might speculate that by supplementing melatonin, carotenoids were more available for the sexual advertisement, not because they were protected from oxidation but because they were less needed by the immune system. Of course this is an appealing alternative explanation that surely deserves further investigation. This could be done by assessing immune functioning, the antioxidant status and the sexual signal of melatonin-supplemented birds. As another way to assess the generality of these preliminary results, it would be interesting to explore whether any of the other non-pigmentary antioxidants (such as vitamins) can also affect the expression of carotenoid-based signals.

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