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RESEARCH ARTICLE

Is oxidative status influenced by dietary carotenoid and physical activity after moult in the great tit (*Parus major*)?

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ABSTRACT

In the context of sexual and natural selection, an allocation trade-off for carotenoid pigments may exist because of their obligate dietary origin and their role both in the antioxidant and immune systems and in the production of coloured signals in various taxa, particularly birds. When birds have expended large amounts of carotenoids to feather growth such as after autumn moult, bird health and oxidative status might be more constrained. We tested this hypothesis in a bird species with carotenoid-based plumage colour, by manipulating dietary carotenoids and physical activity, which can decrease antioxidant capacity and increase reactive oxygen metabolite (ROM) concentration. Great tits were captured after moult and kept in aviaries, under three treatments: physical handicap and dietary supplementation with carotenoids, physical handicap and control diet, and no handicap and control diet. We measured plasma composition (antioxidant capacity, ROM concentration, and vitamin A, vitamin E and total carotenoid concentrations), immune system activation (blood sedimentation) and stress response (heterophil/lymphocyte ratio) and predicted that handicap treatment should influence these negatively and carotenoid supplementation positively. Coloration of yellow feathers was also measured. Carotenoid supplementation increased total plasma carotenoid concentration, decreased feather carotenoid chroma and marginally increased ROM concentration. Handicap increased blood sedimentation only in males but had no clear influence on oxidative stress, which contradicted previous studies. Further studies are needed to investigate how physical activity and carotenoid availability might interact and influence oxidative stress outside the moult period, and their combined potential influence on attractiveness and reproductive investment later during the breeding season.

KEY WORDS: Antioxidants, Carotenoid-based colour, Physiological stress, Immunity

INTRODUCTION

Resource limitations and energetic costs of biological activities do not allow simultaneous maximization of all life history traits, and this leads to physiological trade-offs (Stearns, 1992). For example, the expression of carotenoid-based coloured signals may involve an allocation trade-off of these pigments between signalling, immune defence and antioxidant activity (Faivre et al., 2003). Carotenoids are plant pigments that typically produce red, orange and yellow animal coloration (Hill and McGraw, 2006) in different species, notably in birds (Martín et al., 2008; López et al., 2009; Calsbeek et al., 2010). Vertebrates cannot produce these molecules, and must acquire them from food (Hill and McGraw, 2006; Isaksson, 2009). In addition to their role in pigmentation, carotenoids have multiple physiological roles (Møller et al., 2000; Hill and McGraw, 2006) including regulating and stimulating immune function (Hõrak et al., 2004; Biard et al., 2009; Cote et al., 2010; Hill and Johnson, 2012) and participating in maintaining oxidative balance through their antioxidant properties (Surai and Speake, 1998; Blount et al., 2000; Isaksson, 2009).

According to Sies (1997), oxidative stress is caused by an 'imbalance between oxidants and antioxidants in favour of the oxidants', potentially leading to cellular or tissue damage. Reactive oxygen metabolites (ROMs) i.e. oxidant molecules, are produced by cellular activity or exposure to radiation or pollutants (Finkel and Holbrook, 2000) and may cause a disruption of redox signalling and control, and/or molecular damage (Nikolaidis et al., 2012). Exogenous molecules with antioxidant capacity include some vitamins (e.g. vitamin E) and carotenoid pigments (Sies, 1997). A link between carotenoids and organism antioxidant capacity has been shown in birds (Surai and Speake, 1998; Blount et al., 2000; Isaksson, 2009). The physical and chemical structure of carotenoids allows them to scavenge free radicals (oxidant molecules) by electron absorption and electron transfer reactions (Hill and McGraw, 2006). Hill and Johnson (2012) demonstrated that carotenoids in the endoplasmic reticulum stimulate redox reactions, thereby protecting against oxidative stress. For example, in laying hens or gulls, carotenoid supplementation reduces levels of lipid peroxidation (i.e. oxidative damage) in egg yolk (Surai and Speake, 1998; Blount et al., 2002) and in passerine adults and nestlings plasma carotenoid concentrations were positively correlated with plasma vitamin A and E concentrations (Biard et al., 2005, 2007), which are important lipid-soluble antioxidants (Surai, 1999, 2002). After an immune stimulation, plasma carotenoid concentration decreased in blackbirds *Turdus merula* (Biard et al., 2009) and in zebra finches *Taeniopygia guttata* (Alonso-Alvarez et al., 2004). Moreover, plasma carotenoid concentration was positively correlated with concentrations of lymphocytes and eosinophils in the great tit (Hõrak et al., 2004). Finally, carotenoids also influence body condition. Indeed, in a dietary supplementation experiment, carotenoid-fed blue tit *Cyanistes caeruleus* nestlings were heavier at fledging than control-fed nestlings, and in great tits the influence of carotenoid supplementation on fledging body mass depended on tarsus length (Biard et al., 2006).

It has been shown in several bird species that individuals that ingest more carotenoids or absorb and metabolize them better have brighter yellow plumage (Hill and McGraw, 2006). In the great tit, plasma carotenoid concentration peaks during moult (Del Val et al., 2013), which lasts 3 weeks from mid-June to September in Europe

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(Gosler, 1993; Del Hoyo et al., 2007) and increases yellow feather pigmentation (Isaksson et al., 2007a). Furthermore, carotenoid-based plumage colour in birds has relatively low heritability (Roulin and Ducrest, 2013) and depends more on individual health and environment than on genetic constitution. Indeed, immune challenge (parasitism or infection) decreases the brightness of the yellow throat and ventral colour in wall lizards (Martín et al., 2008; López et al., 2009; Calsbeek et al., 2010). Similarly in birds, immune challenge decreases carotenoid-based yellow bill colour in blackbirds (Faivre et al., 2003), red bill colour of zebra finches (Cote et al., 2010) and feather colour of great tit nestlings before fledging (Romero-Diaz et al., 2013). Thus, coloration may be a reliable signal of physiological or immune condition. However, although many studies document the positive relationship between coloration and body condition or immune system, not all confirm these findings (Hõrak et al., 2004; Isaksson et al., 2005; Geens et al., 2009). Mechanisms underlying seasonal variation in coloration are not yet well understood, but some hypotheses have been advanced, such as UV bleaching, and bacterial or mechanical degradation (Delhey et al., 2010). In great tits, the brightness of the yellow coloration has been shown to increase with the size of the uropygial gland (Galván and Sanz, 2006), which produces an oil that birds deposit on their feathers, its size and oil composition varying with microbiome composition (Jacob et al., 2014). Moreover, activation of the immune system may induce changes in antioxidant status. For example, immune-challenged nestlings of *Falco tinnunculus* showed higher ROM concentration and lower antioxidant capacity (Costantini and Dell'Omo, 2006), and in old zebra finches total antioxidant activity decreased more for immune-activated birds than for control birds whereas the opposite pattern was observed for young birds (Cote et al., 2010). The potential signalling function of carotenoid-based coloration with regard to oxidative stress is still unclear. Indeed, while many studies did not find a significant correlation between oxidative status and coloration (Isaksson et al., 2007b; Isaksson and Andersson, 2008; Cote et al., 2010; Hõrak et al., 2010), others documented a negative correlation between coloration and oxidative damage (Slagsvold and Lifjeld, 1990; Sanz et al., 2000; Leclaire et al., 2011; Patterson et al., 2011) due to the antioxidant activity of carotenoids (Møller et al., 2000).

We investigated the influence of dietary carotenoids on the antioxidant system, using the great tit, *Parus major* Linnaeus 1758, a small passerine common in Europe. This species displays carotenoid-based yellow ventral plumage (Partali et al., 1987) and is insectivorous in spring, caterpillars being the major source of these pigments during the reproductive season and just before moult (Isaksson, 2009), and mainly granivorous (seeds containing few carotenoids) during the rest of the year (Del Hoyo et al., 2007). In order to investigate the role of carotenoids in regulating oxidative stress, we manipulated carotenoid availability through dietary supplementation and measured plasma total antioxidant capacity, ROM concentration as a measure of potential oxidative damage, and concentrations of plasma carotenoids, vitamin A and vitamin E. To be able to examine the effect of carotenoids on oxidative stress, our study was conducted after moult because at this time there are few available carotenoids as a consequence of low insect abundance (Sehhatisabet et al., 2008; Vel'ky et al., 2011), and any stored pigments should have been used for producing yellow feather coloration. Previous studies in species with carotenoid-based plumage colour showed that plasma carotenoid concentration was lower in winter (after moult) than before or during moult, which could be due to lower carotenoid availability and/or to depletion of carotenoid stores during feather growth (Isaksson et al., 2007a; Del Val et al., 2013). Moreover, birds cannot remobilize carotenoids that have already been deposited in feathers, unlike those used for bill or soft tissue coloration (Hill and McGraw, 2006). At the same time, we manipulated physical activity by plucking (or not) three flight feathers from both wings, which should increase physical effort and thereby physiological stress (Slagsvold and Lifjeld, 1990; Sanz et al., 2000; Leclaire et al., 2011; Patterson et al., 2011) and thus modify antioxidant balance. Indeed, intense activity is known to generate oxidative stress (Nikolaidis et al., 2012). A non-linear increase in metabolic rate caused by intense physical activity has been shown to increase ROM concentration by increasing activity in electron-transfer chains and hydrogen escape (Sies, 1997; Metcalfe and Alonso-Alvarez, 2010; Isaksson et al., 2011). Physiological stress is a sign of disturbance of homeostasis (Bradshaw, 2003) and is often reflected by an increase in plasma corticosterone and heterophil concentrations and a decrease in plasma lymphocyte concentration (Ots et al., 1998; Davis et al., 2008). Here, the ratio of heterophils to lymphocytes (hereafter H/Ly) was used as an index of physiological stress (Ots et al., 1998; Davis et al., 2008), and blood sedimentation rate was used as an index of immune system activation (Glick, 2000). Finally, because plumage colour has recently been shown to be a dynamic trait (Galván and Sanz, 2006; Delhey et al., 2010), in order to test for any potential effect of our treatment, we decided to measure feather colour at the beginning and at the end of the experiment. As metabolism is linked with body size (Peters, 1986) and physical activity modifies body condition (Verspoor et al., 2007), body mass and tarsus length were used as covariates.

We predicted that: (1) physical activity would increase ROM concentration and physiological stress and decrease antioxidant capacity; and (2) an increase in dietary carotenoids would increase carotenoid concentration in plasma, immune activation and antioxidant capacity and decrease ROM concentration.

RESULTS

Table 1 presents all mean±s.e. values separately for males, females and treatment groups at the start and at the end of the experiment.

Plasma composition

Total carotenoid concentration was significantly influenced by the interaction between time and dietary carotenoid supplementation ($F_{1,33}$=14.61, P<0.01; supplementary material Table S1; Fig. 1) and by the interaction between time and handicap ($F_{1,33}$=4.67, P=0.03; supplementary material Table S1). *Post hoc* tests showed that carotenoid supplementation increased total plasma carotenoid concentration (all $t_{1,71}$<3.17, P<0.01) and that plasma carotenoid concentration in handicapped and non-handicapped birds did not significantly differ at the end of the experiment ($t_{1,71}$=−2.29, P=11). However, non-handicapped birds had higher total plasma carotenoid concentration at the end of the experiment than other groups had at the start [$t_{1,71}$<−3.23, P<0.01, non-handicapped (h−) end>h− start and handicapped (h+) start; h+ end not being significantly different from other groups].

Variations in plasma total vitamin E and retinol concentrations were not significantly explained by any variable (supplementary material Table S1).

Plasma antioxidant capacity did not differ between carotenoid supplementation groups but did differ between sexes ($F_{1,28}$=17.72, P<0.01; supplementary material Table S2) and between handicapped and control birds in interaction with time ($F_{1,39}$=4.97, P=0.03; supplementary material Table S2). Total plasma antioxidant capacity decreased more over time for the non-handicapped group (*post hoc*

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Table 1. Mean (±s.e.) values for all measured parameters for male and female great tits at the start and at the end of experiment for each treatment group

	Start of experiment			End of experiment		
	h–/c– (7F, 7M)	h+/c– (5F, 8M)	h+/c+ (6F, 8M)	h–/c– (7F, 7M)	h+/c– (5F, 8M)	h+/c+ (6F, 8M)
Total carotenoid concentration (μg ml^{-1})						
Female	1.25±0.11	1.68±0.19	1.21±0.22	1.67±0.41	1.60±0.56	2.07±0.68
Male	1.27±0.28	0.93±0.09	0.95±0.15	1.55±0.16	0.82±0.11	2.15±0.37
Vitamin E concentration (μg ml^{-1})						
Female	0.39±0.16	1.02±0.60	0.12±0.03	0.13±0.04	0.50±0.31	1.63±1.50
Male	0.55±0.43	0.88±0.52	1.56±1.31	0.39±0.25	1.25±0.93	1.21±0.92
Retinol concentration (μg ml^{-1})						
Female	0.12±0.03	0.08±0.01	0.09±0.01	0.16±0.03	0.20±0.04	0.15±0.05
Male	0.16±0.05	0.10±0.02	0.11±0.02	0.53±0.37	0.08±0.02	0.12±0.02
Antioxidant capacity (μmol ml^{-1})						
Female	253±5	271±15	257±7	209±12	232±7	230±9
Male	238±11	226±10	228±12	188±11	205±3	214±8
ROM concentration (μmol ml^{-1})						
Female	1.71±0.33	2.48±0.66	1.39±0.48	2.54±1.74	0.68±0.20	2.59±0.22
Male	1.54±0.72	0.79±0.10	1.44±0.35	0.77±0.25	1.29±0.24	2.88±1.20
Blood sedimentation rate (ratio)						
Female	0.81±0.02	0.75±0.03	0.80±0.03	0.73±0.02	0.73±0.07	0.68±0.03
Male	0.74±0.02	0.80±0.1	0.79±0.02	0.65±0.02	0.73±0.03	0.73±0.02
H/Ly (ratio)						
Female	0.31±0.14	0.11±0.04	0.44±0.13	0.31±0.16	0.09±0.04	0.22±0.05
Male	0.21±0.05	0.18±0.06	0.16±0.07	0.35±0.11	0.26±0.12	0.19±0.07
Lutein absorbance (ratio)						
Female	201±10	173±10	186±16	159±9	165±10	200±11
Male	197±4	184±11	189±5	215±32	207±8	203±8
Carotenoid chroma (ratio)						
Female	0.41±0.02	0.40±0.02	0.39±0.01	0.44±0.02	0.40±0.03	0.34±0.04
Male	0.45±0.01	0.45±0.01	0.46±0.02	0.38±0.06	0.44±0.02	0.43±0.01
UV brightness (ratio)						
Female	0.233±0.004	0.244±0.004	0.237±0.002	0.229±0.004	0.233±0.005	0.230±0.004
Male	0.239±0.004	0.239±0.002	0.240±0.002	0.242±0.003	0.233±0.003	0.230±0.003

ROM, reactive oxygen metabolite; H, heterophil; Ly, lymphocyte; c, carotenoid; h, handicap. Plus and minus signs indicate presence and absence, respectively. The number of females (F) and males (M) used is given in parentheses in the column headings.

test: $t_{1,77}$=5.59, P<0.01) than for the handicapped group (*post hoc* test: $t_{1,77}$=3.94, P=0.01). We found no significant difference between handicap treatments at the start of the experiment (*post hoc* test: $t_{1,77}$=−0.16, P=0.99) and a marginal difference at the end (*post hoc* test: $t_{1,77}$=2.37, P=0.09).

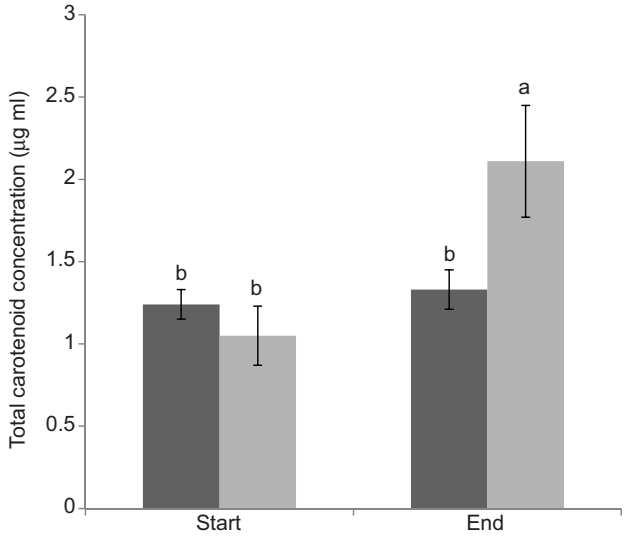

Fig. 1. Total plasma carotenoid concentration as a function of carotenoid supplementation and time. Data are means±s.e.; dark grey, control; light grey, supplemented group. Carotenoid supplementation increased plasma total carotenoid concentration at the end of the experiment. Significant differences between groups are indicated by different letters.

ROM concentration did not significantly differ between handicap treatments, but its variation was explained by the interaction between carotenoid supplementation and time ($F_{1,39}$=3.80, P=0.05; supplementary material Table S2; Fig. 2). At the end of the experiment, birds supplemented with carotenoids had marginally higher ROM concentration than non-supplemented birds (*post hoc* test: $t_{1,39}$=1.95, P=0.06).

At the start of the experiment, plasma carotenoid concentration was positively correlated with antioxidant capacity ($r_{Pearson}$=0.47, $t_{1,37}$=3.22, P<0.01) and was not correlated with ROM concentration ($r_{Pearson}$=0.26, $t_{1,37}$=1.62, P=0.11), whereas antioxidant capacity was positively correlated with ROM concentration ($r_{Pearson}$=0.50, $t_{1,39}$=3.65, P<0.01). Plasma total vitamin E and retinol concentrations were not correlated with antioxidant capacity or ROM concentration (−0.15<$r_{Pearson}$<0.04, $t_{1,39}$<0.30, P>0.30).

At the end of the experiment, however, total antioxidant capacity and carotenoid concentration were no longer significantly correlated ($r_{Pearson}$=0.25, $t_{1,36}$=1.55, P=0.13), ROM concentration and carotenoid concentration were positively correlated ($r_{Pearson}$=0.32, $t_{1,36}$=1.99, P=0.05, n.s. for table-wide significance after Bonferroni correction) and antioxidant capacity was no longer significantly correlated with ROM concentration ($r_{Pearson}$=0.17, $t_{1,39}$=1.06, P=0.29). Plasma total vitamin E and retinol concentrations were not correlated with antioxidant capacity or ROM concentration (−0.07<$r_{Pearson}$<0.13, $t_{1,38}$<0.80, P>0.40).

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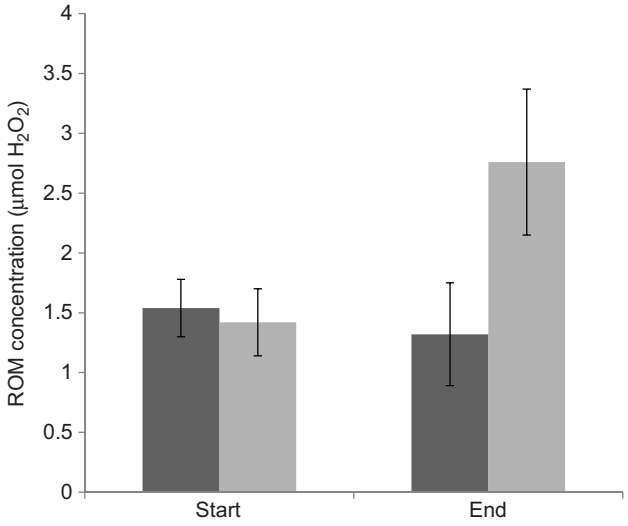

Fig. 2. Reactive oxygen metabolite (ROM) concentration as a function of carotenoid supplementation and time. Data are means±s.e.; dark grey, control; light grey, supplemented group. ROM concentration marginally increased at the end of the experiment for carotenoid-supplemented birds.

Immune system activation and stress response

Blood sedimentation rate (ratio) significantly increased with body mass ($F_{1,38}$=3.71, P=0.05, estimate=0.02±0.01), was lower at the end of the experiment ($F_{1,38}$=20.20, P<0.01, start=0.78±0.01 versus end=0.71±0.01) and was influenced by the interaction between sex and handicap ($F_{1,27}$=8.83, P<0.01; supplementary material Table S3). Handicapped males had a higher blood sedimentation rate than non-handicapped males (Table 1, *post hoc* test: $t_{1,75}$=−3.26, P=0.01) whereas females did not significantly differ between groups (*post hoc* test: $t_{1,75}$=0.95, P=0.78). Non-handicapped males had lower blood sedimentation rates than non-handicapped females (*post hoc* test: $t_{1,75}$=3.43, P<0.01) and handicapped females (*post hoc* test: $t_{1,75}$=−2.59, P=0.06).

The H/Ly ratio was not influenced by handicap but varied significantly with the interaction between sex and carotenoid supplementation ($F_{1,27}$=5.36, P=0.02, Table 1; supplementary material Table S3). *Post hoc* tests revealed that males had a marginally lower H/Ly ratio than females in the supplemented group (*post hoc* test: $t_{1,73}$=1.95, P=0.06) but there was no significant difference between sexes in the other group (*post hoc* test: 0.90>$t_{1,73}$>−1.69, P>0.10).

Colour parameters

Variation in lutein absorbance was significantly explained by the interaction between sex and time (ratio; $F_{1,39}$=4.67, P=0.03; supplementary material Table S4), while carotenoid supplementation and handicap were not retained in the final model. At the end of the experiment, lutein absorbance ratio was significantly higher for males than for females (supplementary material Table S4; *post hoc* test: $t_{1,78}$=−3.12, P=0.01), i.e. males were more yellow than females, whereas at the start of the experiment, values for males and females did not significantly differ (*post hoc* tests: $t_{1,78}$>−1.87, P>0.25).

Larger birds had higher carotenoid chroma (ratio; $F_{1,28}$=7.72, P<0.01, estimate=0.03±0.01; supplementary material Table S4), which also varied with the interaction between dietary carotenoid supplementation and time ($F_{1,37}$=4.36, P=0.04). Surprisingly, breast feathers of supplemented birds had lower carotenoid chroma at the end of the experiment than those of non-supplemented birds (Fig. 3, *post hoc* test: $t_{1,74}$=2.66, P=0.04), i.e. supplemented tits were less yellow than controls.

In the final model investigating variation in UV brightness, both the interaction between handicap and time (ratio; $F_{1,39}$=6.50, P=0.01) and that between handicap and sex ($F_{1,27}$=6.80, P<0.01) were significant. Handicapped birds had lower UV brightness at the end of the experiment than at the start (*post hoc* test: $t_{1,79}$=4.54, P<0.01, Fig. 4) but there was no difference in UV brightness for non-handicapped individuals between the start and end of the experiment (*post hoc* tests: $t_{1,26}$<1.40, P>0.50). Among non-handicapped birds, males had greater UV brightness than females (Table 1, *post hoc* test: $t_{1,76}$=−2.93, P=0.02) but there was no difference between males and females in the handicapped group (*post hoc* test: $t_{1,76}$=0.39, P=0.98).

DISCUSSION

Carotenoids are known to regulate and stimulate immune function (Hõrak et al., 2004; Biard et al., 2009; Cote et al., 2010; Hill and Johnson, 2012) and to participate in reducing oxidative stress (Surai and Speake, 1998; Blount et al., 2000; Isaksson, 2009). We experimentally investigated the influence of dietary carotenoids on oxidative stress in the great tit after moult, by simultaneously manipulating carotenoid availability and physical activity, the latter being an attempt to increase oxidative stress as a result of an increase in metabolism. Our study revealed several results that contradicted our initial predictions. First, increasing physical activity did not affect ROM concentration but increased total antioxidant capacity, prevented the increase in total carotenoid concentration during the experiment and decreased UV brightness (Table 2). Second, although carotenoid supplementation increased total carotenoid concentration as expected, it did not increase antioxidant capacity but slightly increased ROM concentration and decreased a parameter of feather colour, carotenoid chroma (Table 2).

A previous study conducted on birds showed that flight activity increases ROM concentration (Costantini et al., 2013). Indeed, in zebra finches, when flight activity in a test cage was increased by moving perches over a period of 2 h, immediate ROM concentration increased but there was no change in antioxidant capacity, as measured by the concentration of antioxidant enzymes (Costantini et al., 2013). In the present study, flight activity was increased by plucking three primary feathers from each wing. This did not

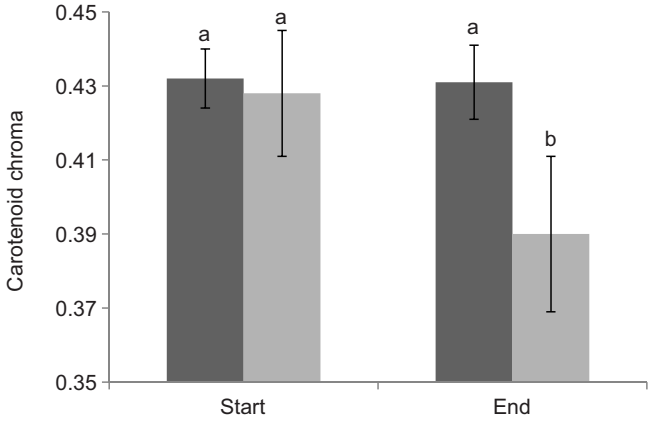

Fig. 3. Carotenoid chroma as a function of carotenoid supplementation and time. Data are means±s.e.; dark grey, control; light grey, supplemented group. Breast feathers of birds in the supplemented group were less yellow at the end of the experiment than they were at the start. Significant differences between groups are indicated by different letters.

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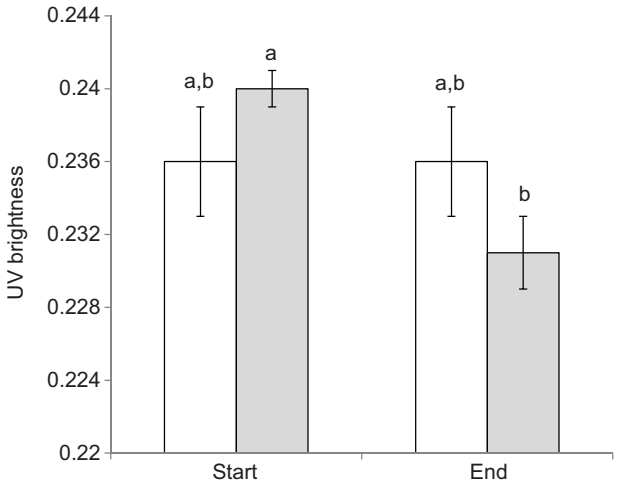

Fig. 4. UV brightness as a function of handicap treatment and time. Data are means±s.e.; white, control; grey, handicapped group. UV brightness of breast feathers decreased during the experiment in handicapped birds whereas it did not change for non-handicapped birds. Significant differences between groups are indicated by different letters.

influence ROM concentration but did influence total antioxidant capacity. Indeed, there was a general decrease in total antioxidant capacity over the course of the experiment, and this decrease was greater for control than for handicapped birds. This suggests that the handicap treatment up-regulated the antioxidant system. However, the absence of a detectable effect on ROM concentration could be due to the time scale. Our study was conducted over 3 weeks and thus measured long-term, and not immediate, variation in ROM concentration (as in Costantini et al., 2013). Moreover, food was given *ad libitum* and might be more caloric and/or richer in antioxidants, than that found in natural conditions at that time of year (Sanz et al., 2000; Patterson et al., 2011), which could have buffered the expected effect of plucking some wing feathers on ROM concentration. Birds may not have been significantly constrained with regard to the availability of energy or antioxidants that are needed to activate repair pathways (Finkel and Holbrook, 2000; Larcombe et al., 2008, 2010) and may have thus overcome the physical handicap without increasing ROM concentration. However, the effects of physical activity on ROM concentration may also depend on other factors such as age (Finkel and Holbrook, 2000; Costantini et al., 2008), or pollutants (Koivula and Eeva, 2010). Moreover, we found that in males, but not in females, physical handicap increased blood sedimentation rate. It has been reported that in birds, females have a higher blood sedimentation rate than males (Glick, 2000; but see Svensson and Merilä, 1996), which was indeed the case at the start of the experiment. The higher female blood sedimentation rate may be related to a higher haematocrit of female than male great tits (Hõrak et al., 1998; Glick, 2000; Kilgas et al., 2006; but see for other species Fair et al., 2007). This may have rendered males more sensitive to the handicap treatment than females, whose blood sedimentation rate was not significantly affected over the course of the experiment.

Table 2. Summary of the effects of carotenoid supplementation and physical handicap treatment on plasma composition, antioxidant system, immune and physiological stress, and feather colour in adult great tits

	Carotenoid supplementation	Physical handicap
Total carotenoid concentration	+	–
Vitamin E concentration	0	0
Retinol concentration	0	0
Antioxidant capacity	0	+
ROM concentration	(+)	0
Blood sedimentation rate	0	0
H/Ly ratio	0	0
Lutein absorbance	0	0
Carotenoid chroma	–	0
UV brightness	0	–

'0' indicates no significant effect, a minus sign indicates a significant negative effect, a plus sign in parentheses indicates a slightly positive effect and plus sign indicates a significant positive effect.
ROM, reactive oxygen metabolite; H, heterophil; Ly, lymphocyte.

Physical handicap decreased breast feather UV brightness during the experiment. Variation in UV colour is determined by feather structure and quality (Griffith et al., 2003; Limbourg et al., 2004, 2013) but also by the presence or composition of uropygial gland secretions or the community of feather bacteria or mites (Hill and McGraw, 2006; Pérez-Rodríguez et al., 2011). For example, when preen oil was removed from feathers of Bohemian waxwings (*Bombycilla garrulus*), UV chroma increased, suggesting a direct effect of preen oil on structural colour (Pérez-Rodríguez et al., 2011). Feather plucking may thus have modified uropygial gland function and/or induced an infection, as feather mite and microbiome abundance have been shown to influence uropygial gland size and function (Galván and Sanz, 2006; Jacob et al., 2014), and handicapped males showed higher blood sedimentation rate. However, the effect of the handicap treatment on UV chroma was similar in females and males, though the former showed no parallel effect on blood sedimentation. This suggests the effect of the handicap treatment may have been mediated by a change in preening behaviour and an increase in the deposition of uropygial secretions to feathers.

Surprisingly, dietary carotenoid supplementation directly or indirectly decreased an aspect of yellow plumage coloration, carotenoid chroma, during the experiment. Feathers are passive structures and variation in their coloration is due to pigment composition and concentration, which reflects the type and quantity of carotenoids ingested during moult (Hill and McGraw, 2006; Peters et al., 2007). Feather coloration may also change after moult (Galván and Sanz, 2006; Delhey et al., 2010). Few studies have explored variation in carotenoid-based colour after moult (Hill and McGraw, 2006; Peters et al., 2008; Delhey et al., 2010). Previous studies have experimentally shown that feathers exposed to sunlight become less yellow (Surmacki, 2008; Surmacki et al., 2011) probably due to an effect of pigment oxidation caused by photon absorption. In our experiment, carotenoid supplementation increased ROM concentration in plasma and may also have influenced other parameters that were not measured here, such as uropygial secretions, which may have indirectly induced the observed decrease in carotenoid chroma. Our results show that dietary carotenoid availability also affected yellow plumage coloration after moult, which, to our knowledge, has never been shown before. Although the mechanism is as yet unknown, as females may select more colourful males (Gosler, 1993; Hill and McGraw, 2006), and dietary carotenoid availability after moult may also influence partner choice in the next reproductive season.

Previous studies on birds have shown that carotenoid-supplemented females produced eggs with higher carotenoid concentration and lower lipid peroxidation in yolk (Surai and Speake, 1998; Blount et al., 2002), with a negative correlation

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between carotenoid concentration and lipid peroxidation in plasma (Woodall et al., 1996; Hõrak et al., 2007). However, we did not find the expected increase in total antioxidant capacity or decrease in ROM concentration with carotenoid supplementation. We even found a slight ROM concentration increase. There are two possible explanations for these counterintuitive results. First, the marginal increase in ROM concentration might be explained by an inappropriately high dose of supplementary antioxidant (Hill and McGraw, 2006; Hill and Johnson, 2012). We can reject this hypothesis because blood assays revealed that total carotenoid concentration in the plasma of supplemented birds was within the natural range and lower than previously reported in the same and/or closely related species during the breeding period (Hõrak et al., 2004; Biard et al., 2005; Isaksson et al., 2007a) and in autumn (Isaksson et al., 2007a). Another possibility could be that increased availability of carotenoids in the diet might promote short-term benefits for the antioxidant system through decreased ROM concentration (Larcombe et al., 2008) but might also induce a long-term effect on ROM concentration by lowering the stimulation of antioxidant capacity (Metcalfe and Alonso-Alvarez, 2010). Metcalfe and Alonso-Alvarez (2010) proposed that reactive oxygen species could be regulating agents that continually stimulate antioxidant defences to allow an efficient response in the case of an important oxidative challenge.

In summary, physical activity did not increase ROM concentration but increased antioxidant capacity and decreased breast feather UV reflectance, while carotenoid supplementation decreased breast feather carotenoid chroma and slightly increased ROM concentration. Complex influences of dietary carotenoid and physical activity on ROM concentration were uncovered, and further research should seek to elucidate how physical activity may or may not increase ROM concentration depending on diet quality. Moreover, how diet quality affects the change in yellow colour once feathers are fully grown and whether this could be mediated by oxidative stress is still unclear and deserves further study. Finally, our results show that the availability of carotenoids in the diet is not only essential during moult for the development of coloured signals but may also play a role outside this period, with potential consequences for sexual attractiveness and reproductive investment a few months later.

MATERIALS AND METHODS

This study was conducted in full compliance with French laws and regulation, including recommendations for the ethical treatment of experimental animals in France.

Biological model and rearing conditions

The great tit is a small common insectivorous passerine found in Europe except at high altitudes and latitudes. This species breeds in artificial cavities such as nest boxes, so its demography, life-history and behaviour have been intensively studied in natural populations (Hõrak et al., 2004; Biard et al., 2006; Quesada and Senar, 2007; Dauwe and Eens, 2008; Isaksson, 2009; Evans and Sheldon, 2013).

Great tits were caught with mist nets from 22 to 31 November 2012 in a private forest in a rural area located 80 km south of Paris, at the Centre de Recherche en Ecologie Expérimentale et Prédictive (CEREEP, 48°17′N, 2°41′E). Twenty-three males and 19 females were caught and kept in outdoor aviaries (3×3×4 m), four adults in each of nine aviaries and two adults in each of three aviaries. One female died before the start of the experiment but no deaths occurred during the experiment. Adults captured the same day were assumed to belong to the same winter flock and therefore were housed together in the same aviary independent of the sex ratio. Indeed, outside the breeding season, passerines form roaming flocks (Aplin et al., 2012) and we preferred to maintain previously established groups in order to avoid fights in the aviaries. However, there were always members of several flocks, captured on the same day, mixed in each aviary. Birds were fed with mealworms (3.4±4 mg kg^{-1} of β-carotene, 0.2±0.3 mg kg^{-1} of lutein and no zeaxanthin; Oonincx and Dierenfeld, 2012) mixed with a premix of proteins, vitamins, minerals, amino acids and trace elements (NutriBird A19 Versele Laga), sunflower seeds (containing 1 mg kg^{-1} carotenoids; Peters et al., 2008), and crushed peanuts mixed with a premix of fat components, vitamins, minerals, amino acids and trace elements (Orlux Uni Pâtée Premium Versele Laga, containing 3.65 mg kg^{-1} of β-carotene as indicated by the manufacturer). Water and food were given *ad libitum*. Water was changed every day and food was changed every 3 or 4 days. Birds were held in captivity between 1 and 2 weeks before the onset of the experiment to allow acclimatization. Each outdoor aviary contained many freshly cut branches and nest boxes (one for each bird).

On the day of capture, individuals were individually identified with a numbered aluminium ring (National Museum of Natural History), tarsus length was measured and eight breast feathers were taken to measure plumage reflectance (see 'Plumage coloration').

Experimental design

We used a crossed design in which dietary carotenoid availability and physical activity were manipulated. A solution of carotenoids was added to the drinking water to a final concentration of 50 μg g^{-1}: 0.2 g of FloraGlo (5% lutein and 0.86% zeaxanthin, DSM France) in 200 ml of water. During the acclimatization phase, we observed that great tits drank about 5 g of water per day. In addition, over the course of the experiment, we observed that less than 20 g of water was drunk per day in each aviary (i.e. by two to four birds altogether). With a concentration of 50 μg g^{-1} carotenoids in the drinking water, birds therefore ingested a total of 50 to 500 μg carotenoids per day. In the same species, carotenoid concentration in plasma has been recorded at about 30 μg ml^{-1} for a body mass of around 18 g (Hõrak et al., 2004; Isaksson et al., 2007a); thus, the total quantity of circulating carotenoids could be estimated as 54 μg considering blood to represent 10% of body mass (Sturkie, 1986). In addition, it is known from supplementation experiments in poultry that the efficiency of carotenoid absorption in birds is about 20% of the used dose (Surai et al., 2001; Surai, 2002). Daily carotenoid intake should therefore have stayed within a physiological and natural range. Water was changed every day in all experimental groups at the same time of the day (11:00 h). There was no difference in water consumption between birds given carotenoid-supplemented water and control water (M.V., personal observation).

In order to induce a physical handicap, flight ability was decreased by plucking the fifth, seventh and ninth primary feathers from both wings (Chai, 1997; Verspoor et al., 2007). Plucking these feathers has been shown to decrease body condition (Sanz et al., 2000; Patterson et al., 2011; but see also Love and Williams, 2011). Plucking feathers induces their regrowth, whereas clipping does not, so our treatment manipulated flight ability, and consequently physical activity, and at the same time increased energy demand for feather growth (Sturkie, 1986; Chai, 1997).

One week prior to the experiment, the adults were divided into three experimental groups: (i) carotenoid supplementation and physical handicap (c+/h+), (ii) no dietary supplementation with physical handicap (c−/h+) and (iii) control with no supplementation and no handicap (c−/h−). The experimental design could not be fully crossed and the treatment c+/h− was missing because of the lower than expected number of birds captured. Three aviaries with four adults (except for one aviary with three adults in c−/h+) and one aviary with two adults per treatment were used. There were eight males and six females in c+/h+, eight males and five females in c−/h+ and seven males and seven females in c−/h−. Given that groups were composed before the start of the experiment, some aviaries had a biased sex ratio due to the respected flock structure. However, sex ratio was similar in each treatment.

On the first experimental day, all birds were recaptured with mist nets (four bags 210/1 16 mm mesh, Bonardi, Italy) in the aviaries. Blood samples were taken (see 'Laboratory analyses', below), body mass was measured and for experimental groups with handicap, the three wing feathers were plucked from each wing.

We ran the experiment for 21 days to ensure a sufficient feather growth without reducing the time left in the season for prospecting reproductive

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habitats (Isaksson and Andersson, 2008). At the end of the experiment, a blood sample, a breast feather sample and morphometric measures (tarsus length and body mass) were taken. Variation in feather regrowth, measured using pictures of the right wing taken at the end of the experiment, was not explained by morphology values (tarsus length: $F_{1,19}$=2.06, P=0.17, and initial body mass: $F_{1,19}$=0.06, P=0.94), sex ($F_{1,19}$=0.23, P=0.64) or carotenoid supplementation ($F_{1,19}$=0.52, P=0.45). Great tits were released at the place where they had been caught.

Laboratory analyses

Blood samples of up to 250 µl were taken at the beginning and the end of the experiment from the brachial vein in heparinized capillary tubes for haematocrit, and a 1 or 2 µl drop of blood was smeared on a microscope slide for blood cell counts. Blood samples were placed at 4°C and left upright to measure sedimentation after 8 h, i.e. the ratio of red blood cells to total blood volume, as an index of immune system activation (Glick, 2000; Campbell and Ellis, 2007). Indeed, high sedimentation rate reflects acute infections and inflammatory disease as the number of leucocytes increases due to stress and infection (Glick, 2000; Campbell and Ellis, 2007), leading to elevated levels of immunoglobulins and fibrinogen in the blood (Sturkie, 1986; Svensson and Merilä, 1996). Blood samples were centrifuged for 8 min at 1000 rpm, and plasma was extracted and kept at −20°C until later laboratory analyses of antioxidant capacity.

Smears were air-dried, fixed in absolute methanol and stained with Giemsa (Sigma Giemsa, modified, 0.4% w/v) (Biard et al., 2010), then examined under oil immersion at 1000× magnification to count the proportion of different types of leucocytes in a total of 50 leucocytes per smear in random order (by the same person, M.V.). A total of only 50 leucocytes could be counted (and not 100 as in other studies; Ots et al., 1998; Biard et al., 2007; Peters et al., 2007; Verboven et al., 2009) because of a lack of leucocytes (mean±s.e. 26±17 leucocytes per examination of 10,000 erythrocytes, as compared with other studies in great tits: 63±3, Peters et al., 2007; and 50±23, Ots et al., 1998). Leucocyte counts can vary among populations (Kilgas et al., 2006; Davis et al., 2008), and the stress of captivity or seasonality can cause low leucocyte counts (Hõrak et al., 1998; Davis et al., 2008; Bókony et al., 2012), but this is an unlikely explanation for this observation because we have found similar leucocyte counts in this same population during the reproductive period. Repeatability scores of our counts were highly significant (H/Ly ratio $F_{91,92}$=8.28, P<0.01, intraclass correlation coefficient=0.78; Lessells and Boag, 1987). A high H/Ly ratio is often interpreted as a sign of physiological stress (Ots et al., 1998; Lobato et al., 2005; Campbell and Ellis, 2007) and, for example, sustained flight has been shown to increase the H/Ly ratio (Matson et al., 2012).

The level of oxidative stress was measured through plasma total antioxidant capacity and ROM concentration. The total antioxidant capacity test (OXY-Adsorbent test, Diacron, Grosseto, Italy) assesses the antioxidant capacity of plasma including both exogenous and endogenous antioxidants by measuring the ability to resist a massive oxidant attack of hypochlorous acid (HOCl). Plasma (3 µl) was diluted 1:100 with distilled water; 5 µl of the diluted plasma was incubated with a 200 µl aliquot of a HOCl solution for 10 min at 37°C. Then, 5 µl of *N*,*N*-diethyl-paraphenilenediamine, which is oxidized by the residual HOCl and transformed into a pink derivative, was added. The absorbance was read at 505 nm and values were calculated according to a calibration curve run alongside each essay. Total antioxidant capacity was expressed as µmol of HOCl neutralized ml^{-1} (Costantini et al., 2011). A low value indicates a low antioxidant capacity. Samples were measured twice and were found to be highly repeatable ($F_{161,162}$=45.1, P<0.01, intraclass correlation coefficient=0.96). Mean values were used in this study.

The ROM concentration test (d-ROMs test, Diacron) measures the concentration of hydroperoxides, which derive from the oxidation of several molecular substrates (Miller et al., 1993; Beckman and Ames, 1998). Plasma (4 µl) was diluted 1:100 with acetate buffer (pH 4.8), mixed with 2 µl of *N*,*N*-diethyl-paraphenilenediamine and incubated for 75 min at 37°C. The acidic pH favours the release of iron and copper ions from plasma proteins. These metals catalyse the cleavage of ROOH, leading to the generation of alkoxyl and alkyperoxyl radicals, two highly reactive and histolesive pro-oxidants. When these compounds react with *N*,*N*-diethyl-paraphenilenediamine they produce a complex whose intensity of pink is directly proportional to their concentration. The absorbance was read at 505 nm. The concentration of ROMs was calculated according to a calibration curve obtained by measuring the absorbance of a standard solution and expressed as µmol of H_2O_2 equivalents. The concentration of ROMs in two replicated subsamples was highly repeatable ($F_{82,83}$=1044.8, P<0.01, intraclass correlation coefficient=0.99).

Carotenoids and vitamins A and E were extracted and identified by high performance liquid chromatography (HPLC) as described elsewhere (Ewen et al., 2006). In brief, 20 µl of plasma was homogenized with 40 µl of ethanol and then twice extracted by centrifugation with 40 µl of hexane. After that, hexane extracts were pooled and evaporated at 60–65°C under a flow of nitrogen and the residue was dissolved in 100 µl of a 50:50 mix of dichloromethane and methanol. Total carotenoid concentration of each sample was quantified by injecting 25 µl of purified extract into a Spherisorb type S5NH2 5 µm C18 reverse-phase column (250×4.6 mm, Phase Separation Ltd, Clwyd, UK) with a mobile phase of methanol:distilled water (97:3) delivered at a flow rate of 1.5 ml min^{-1} (using Thermo Spectra System P1000 isocratic pumps), and detected with a UV/visible absorbance detector at 445 nm (using Thermo Spectra System P100 isocratic pumps). The HPLC was calibrated using lutein standards (DSM France; relative standard deviation, RSD%=0.60 at 1 ppm, 0.71 at 2 ppm at 0.28 at 4 ppm; Rachieru et al., 2009). Vitamin A (retinol) and vitamin E (α-, δ- and γ-tocopherol) concentrations were detected with a Spherisorb type ODS2 3 µm C18 reverse-phase column (150×4.6 mm Phase Separation Ltd), by injecting 25 µl of extract with a mobile phase of methanol:distilled water (97:3) at a flow rate of 1.05 ml min^{-1} using fluorescence detection for 295 nm wavelength excitation and 330 nm wavelength emission for vitamin E and 330 nm excitation and 480 nm emission for vitamin A. Peaks of retinol, α-, δ- and γ-tocopherol were identified by comparison with the retention time of tocopherol standards (Sigma, Poole, UK), which were also used to calibrate the HPLC (retinol: RSD%=1.49 at 1 ppm, 0.76 at 2 ppm and 0.28 at 4 ppm and α-tocopherol: RSD%=1.88 at 0.5 ppm, 1.79 at 1 ppm and 0.37 at 2 ppm).

Plumage coloration

Four replicate reflectance spectra were obtained from two sets of four feathers from each bird arranged in random order (Quesada and Senar, 2006), using a USB 2000+ spectrometer with a DH 2000 deuterium–halogen light source (Ocean Optics, IDIL, Lannion, France) (Biard et al., 2005). We used a bifurcated fibre-optic probe (Ocean Optics QR200-7-SR-BX Premium 200 µm) on which a cylindrical black plastic sheath was mounted to exclude ambient light and standardize measuring distance. Reflectance was calculated relative to a white standard (Ocean Optics spectralon WS-1-SL) with SpectraSuite software (Ocean Optics). Every three individuals, a dark and a white standard scan were obtained. Samples were taken randomly with respect to individual, aviary, treatment and time of sampling.

Three different parameters describing the variation in spectral shape were calculated for each spectrum. As we were interested in the colour produced by carotenoids, we quantified the relative reflectance around wavelengths of carotenoid peak absorbance, referred to as carotenoid chroma, as the height of the peak in the yellow band divided by reflectance in the yellow band: $(R_{700}-R_{450})/R_{700}$ (Peters et al., 2007). As lutein is the main carotenoid present in great tit breast feathers (Örnborg, 2002; Isaksson and Andersson, 2007; Isaksson et al., 2007a), we measured lutein absorbance, calculated from mean reflectance in the 445–455 nm interval as −log10(mean reflectance) (Hõrak et al., 2001). Carotenoid chroma and lutein absorbance increase when carotenoid (lutein, zeaxanthin, β-carotene, violaxanthin and neoaxanthin) or lutein content in feathers increases (Hill and McGraw, 2006), thereby giving yellower feathers (Hõrak et al., 2001; Örnborg, 2002; Peters et al., 2007). Feather structure, which causes variation in UV reflectance, is also a reliable cue of individual quality (Griffith et al., 2003; Limbourg et al., 2004, 2013). We thus calculated 'UV brightness' as the proportion of UV light reflected (300–400 nm reflectance divided by total reflectance, 300–700 nm).

Repeatability of parameters within individuals and within sets of feathers within individuals, calculated as the intraclass correlation coefficient, was highly significant [within sets of feathers (4 times): P<0.01, $F_{173,523}$>7.62, $r_{Pearson}$>0.55; within individuals (8 times): P<0.01, $F_{86,610}$>10.65, r>0.65]. For

each individual we calculated carotenoid chroma, lutein absorbance and UV brightness, which were not inter-correlated ($P>0.10$, $-0.27<r_{Pearson}<0.06$), at the start and at the end of the experiment.

Statistical analyses

All statistical analyses were performed in R v. 2.15.0 (R Development Core Team, 2014). We used linear mixed models with backward elimination factors by Akaike's information criterion to select the best predictive model. Final models were maximum-likelihood (REML) linear mixed models, run using the function lme in the package nlme and the function Anova in the package car, with type III sum of squares. Total carotenoid, retinol and total vitamin E plasma concentrations and H/Ly ratio were log-transformed. No residuals of final models deviated from a normal distribution.

All statistical models included aviaries and individual identity as random factors, which were not significant for any model (aviaries: $Z_{1,12}<1.06$ and $P>0.14$, and individual identity: $Z_{1,41}<1.28$ and $P>0.20$). According to predictions 1 and 2, in order to test the effects of physical activity and dietary carotenoid availability on the antioxidant system (total antioxidant capacity, ROM concentration, total carotenoids, vitamin A and vitamin E), on the stress response (blood sedimentation and H/Ly ratio) and on plumage colour (lutein absorbance, carotenoid chroma and UV brightness), all models included as fixed effects tarsus length and body mass, and three-way interactions among carotenoid supplementation or physical handicap with sex and time of experiment (start or end of the experiment). When final models included significant interactions, differences of least squares means were tested between levels of treatments or sex with adjusted values of P, using the Tukey–Kramer method in SAS v. 9.3 (SAS Institute Inc., Cary, NC, USA).

Means are presented ±s.e. At the start of the experiment, plasma composition (antioxidant capacity, ROM concentration, and vitamin A, vitamin E and total carotenoid concentrations), immune system activation (blood sedimentation), stress response (H/Ly ratio), colour and morphological values were not significantly different among groups or aviaries ($F_{1,11}<2$ and $P>0.05$). Aberrant values were deleted for ROM concentration (two values higher than 10 µmol of H_2O_2 equivalents). One male was not weighed at the end of the experiment. Total carotenoid concentration was missing for two individuals at the start and two others at the end of the experiment because of insufficient plasma. Retinol and vitamin E values were missing for one female at the end of the experiment for the same reason.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.V. designed the experiment, captured, manipulated and fed birds in aviaries, carried out antioxidant system and blood smear analyses, and statistical analyses, and wrote the article. B.D. and S.P. captured and fed birds in aviaries. F.K. ran carotenoid and vitamin analyses. S.M. and C.B. designed the experiment, captured and manipulated birds, helped with statistical analyses and wrote the article.

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Supplementary material

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