Age-dependent allocation of carotenoids to coloration *versus* antioxidant defences

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SUMMARY

Aging is commonly attributed to age-related changes in oxidative damage due to an increased production of reactive oxygen species (ROS) and a weakened efficacy of enzymatic antioxidants. These age-related changes might therefore modify the use of dietary antioxidants, including carotenoids. As carotenoids are closely associated with the expression of secondary sexual signals, the allocation of carotenoids to sexual signal *versus* antioxidant defences may vary with age. In this study, we explored how carotenoid-based ornament and antioxidant activity varied with age and how an inflammatory-induced oxidative burst affected ornament and antioxidant activity across a range of ages. Using zebra finches (*Taeniopygia guttata*) as a model species, we assessed circulating carotenoids, beak coloration and the plasma antioxidant status of birds of different ages before and after an inflammatory challenge. Our results show that old individuals display similar carotenoid-based sexual signals regardless of the availability of circulating carotenoids, suggesting a terminal investment of old individuals in their last reproductive event. Additionally, we found that an inflammatory insult induced a decrease in the total antioxidant activity and in the expression of a carotenoid-based sexual signal in the oldest individuals. These results suggest that old individuals pay an extra cost of immune activation possibly because the efficiency of antioxidant machinery varies with age.

Key words: aging, honesty, signal, allocation strategies, zebra finches.

INTRODUCTION

Aging refers to the age-related decline in biological functions ending up in decreased fitness features (Beckman and Ames, 1998; Kirkwood, 2002; Kregel and Zhang, 2007; Weinert and Timiras, 2003). According to the disposable-soma theory of the evolution of senescence, organisms have to face a decision about the optimal allocation of metabolic resources between self-maintenance and reproduction (Kirkwood, 1977; Kirkwood and Rose, 1991). When facing high levels of extrinsic mortality, the optimal resource allocation should be to invest fewer resources towards the maintenance of somatic structures than are required for an extended life span (e.g. Carranza et al., 2004).

From a proximal point of view, aging is attributed to age-related changes that increase the risk of cellular death (Finkel and Holbrook, 2000; Harman, 1956; Harman, 1981). There is now accumulating evidence that age-related changes are due to oxidative damage (Finkel and Holbrook, 2000; Wickens, 2001). Oxygen and nitrogen reactive species (ROS and RNS) are unstable molecules that can damage proteins, lipids and DNA, finally compromising cellular function and integrity (Finkel and Holbrook, 2000; von Schantz et al., 1999; Wickens, 2001). To counteract the negative effect of ROS and RNS, aerobic organisms have evolved a variety of defence mechanisms, including enzymatic [superoxide dismutase (SOD), catalase (CAT) and peroxidases (GPX)] and non-enzymatic scavengers mostly acquired with food [carotenoids, vitamins E and C (Finkel and Holbrook, 2000; Harman, 1995; Wickens, 2001)]. Oxidative damage results from the unbalance between ROS/RNS production and the availability of antioxidant defences (Halliwell and Gutteridge, 2007). Age can alter both sides of this balance since aging has been associated with both an increase in ROS production and a weakened efficacy and/or availability of enzymatic antioxidants (Alonso-Alvarez et al., 2006; Beckman and Ames, 1998; Finkel and Holbrook, 2000; Torres and Velando, 2007; Wickens, 2001). Consequently, one might predict that senescent

individuals should use more dietary antioxidants to counteract the increase in ROS/RNS production. However, the way that dietary ROS scavengers are allocated to the antioxidant function depending on individual age has been largely neglected in the evolutionary biology literature. This is surprising, given that some of the dietary antioxidants are also closely associated with other fitness-linked traits, such as the expression of secondary sexual signals.

In recent years, carotenoids have been mastered as examples of compounds with pleiotropic effects. Carotenoids are indeed necessary for the development of yellow to red coloured ornaments, to stimulate the immune response, and have been shown to play a role as antioxidants (Alonso-Alvarez et al., 2004; Bendich, 1989; Blount et al., 2002; Horak et al., 2007; Pike et al., 2007). Nevertheless, it is important to keep in mind that the physiological properties of these pigments are currently under debate, since not all studies have provided support for the antioxidant function of carotenoids in vivo (Costantini and Moller, 2008; Hartley and Kennedy, 2004; Isaksson et al., 2007). For instance, Pike et al. (Pike et al., 2007) have shown that carotenoid-supplemented male sticklebacks (*Gasterosteus aculeatus*) have a more exuberant nuptial coloration, a better survival rate, a higher reproductive output, and, more importantly, a reduced level of oxidative stress compared with control individuals, whereas Costantini et al. (Costantini et al., 2007) did not report any effect of carotenoid supplementation on oxidative stress of nestling kestrels (*Falco tinnunculus*). It is not immediately clear why different studies have reported different results. Although, this is beyond the scope of the present article, it is possible that context-dependent effects (e.g. dose and duration of the supplementation, environmental conditions, additive/interactive effects with other antioxidants) might explain some of this variability.

Since carotenoids cannot be synthesized *de novo* by animals (Goodwin, 1984), their supposed multiple functions should create trade-offs in carotenoid-limited individuals between ornamental

pigmentation, immune and oxidative protection (Blount et al., 2003; Faivre et al., 2003b; McGraw and Ardia, 2003). The optimal allocation of carotenoids to these different functions is likely to depend not only on the particular environmental conditions (presence of parasites and of environmental stressors, intensity of sexual selection) faced by an individual but also on its physiological status. Once again, age can alter the optimal allocation of carotenoids to conflicting functions by changing the physiological requirements (age-associated pro-oxidant status). Moreover, given that carotenoid-based sexual signals have been suggested to convey information about the health and oxidative status of their bearers (Lozano, 1994; von Schantz et al., 1999), one might expect that older males should have less exuberant signals than younger, healthier individuals (Torres and Velando, 2007). Alternatively, older individuals might contribute an ultimate allocation to the production of sexual signals at the expense of maintenance functions, as a form of terminal investment (Bonneaud et al., 2004; Clutton-Brock, 1984; Velando et al., 2006). For example, Pike et al. (Pike et al., 2007) showed that non-supplemented sticklebacks (*Gasterosteus aculeatus*) allocate carotenoids to nuptial coloration at the expense of their health and might trade-off future reproduction opportunities for a large investment in current reproduction. This hypothesis also fits theoretical models predicting an increase in the signal intensity with age to maximize the reproductive investment of old individuals relative to younger individuals (Kokko, 1997). To our knowledge these questions have been poorly explored.

The aim of this study was to investigate the age-related allocation of carotenoids to sexual signal versus antioxidant defences in zebra finches (*Taeniopygia guttata*). Using an experimental approach, we explored (1) how carotenoid-based ornament and antioxidant status vary with age and (2) how an oxidative burst affects ornament and antioxidant status across a range of ages. To achieve this goal, we assessed circulating carotenoids, beak coloration and plasma antioxidant status in birds of different ages before and after an inflammatory challenge.

MATERIALS AND METHODS
General procedure
The study was carried out on 65 captive zebra finches (*Taeniopygia guttata* Veillot) held in indoor cages (0.6 m×0.4 m×0.4 m) with food (commercial seed mix) and water provided *ad libitum*. Temperature (21±1°C) and daily light cycles (13 h:11 h, L:D) were controlled during the experiments. Birds were obtained from two bird breeders who have kept the birds under optimal conditions with two to three breeding events per year for each one-year-old and older birds, and no breeding attempt for 5-month-old birds. The birds were acclimatized for 2 months before the experiments. Each individual wore a ring with information about its origin and birth year. Birds were sexually mature with ages that spanned from <1 year to 6 years [0–1 year old: 10 birds (four females, six males), 1–2-years old: 14 birds (eight females, ix males), 2–3 years old: 21 birds (nine females, 12 males), 3–4 years old: six birds (six females), 4–5 years old: nine birds (six females, three males), 5–6 years old: five birds (two females, three males)]. Two individuals of the same sex (one of each immune treatment) were kept per cage with a separation in the middle to ensure that each bird was isolated. Cages were randomly distributed in the experimental room. For each bird, beak colour was measured at the beginning and at the end of the experiment, 3 weeks later (day 0 and day 21, respectively). Body mass (to the nearest 0.1 g) was also measured at day 0, day 1, day 15 and day 21. For each individual, a blood sample (~100 μl) was collected at the beginning of the experiment, immediately before the first immune challenge (day 0), 1 day later (day 1±1 h), and at the end of the experiment (day 21). Blood samples were collected from the brachial vein using sterile needles and heparinized capillaries. Afterwards, samples were centrifuged for 15 min at 1800 *g* at 4°C, and the plasma was separated and stored at –80°C. Experiments were carried out in compliance with national legal requirements and permission (B. F. permit no. 21-CAE-085).

Immune activation
At day 0, birds were assigned to one of two treatments, with respect to their age and sex. Half of the birds were injected intraperitoneally with a mixture of antigens (total volume injected was 0.1 ml) known to strongly activate the inflammatory response and generate an oxidative burst: 0.04 ml of a solution of lipopolysaccharides (LPS) (*E. coli* serotype O55:B5; Sigma, St Louis, MO, USA) diluted in phosphate-buffered saline (PBS; 0.375 mg ml^{-1}); 0.04 ml of a solution of peptidoglycan (*Staphylococcus aureus*; Sigma; 0.375 mg ml^{-1} in PBS); 0.02 ml of Complete Freund's adjuvant [FCA, each ml containing 1 mg of *Mycobacterium tuberculosis* (H37Ra, ATCC25177) Sigma]. This treatment triggers the recognition of different pathogen-associated molecular patterns by the immune system of the host, and then stimulates different signalling pathways and branches of the inflammatory process (Akira et al., 2006; Janeway and Medzhitov, 2002). Therefore, it mimics infection with a large array of pathogens (e.g. gram-positive and gram-negative bacteria) as may occur in a pathogen-rich environment. The remaining birds were injected with the same volume (0.1 ml) of PBS as a control. In order to maintain the immune activation during the 3 weeks of the experiment, birds were injected once per week (day 0, day 7, day 14). Repeated injections over several weeks aimed to simulate a chronic infection status over a period required to observe a change in bill colour, as already applied in other studies (Eraud et al., 2009; Gautier et al., 2008).

Measurement of bill colour
Bill colour was assessed using pictures taken with a digital camera (Canon EOS3) with a Repro lighting unit (ARIC TF6, Kaiser Fototechnik, Buchen, Germany) and black curtains controlling light conditions. The colour was analyzed with Lucia G 4.81 software (Nikon). Hue (i.e. redness) of the upper surface of the bill was determined as the mean value of the whole bill surface (32±0.8 mm^2). Bill colour measurements were always performed by the same person (J.C.) blindly with respect to the treatments. This colour parameter (low values of hue indicate redder colour) has already been extensively used to measure variation in bill colour in zebra finches (Birkhead et al., 1998; Blount et al., 2003; Burley et al., 1992) and is highly repeatable [two repeats on 10 individuals; $F_{9,10}$=11.37; P=0.0004; r=0.90 (Lessells and Boag, 1987)].

Assessment of total antioxidant activity
Several relevant components of antioxidant defences could have been assessed. However, limited amounts of blood were collected for ethical reasons, and we have chosen a method designed to assess total antioxidant activity of the plasma, including potential effects of circulating carotenoids. Total antioxidant activity (TAA) was measured in samples of plasma using a decolourization assay (Re et al., 1999). The assay measures the rate at which a pre-formed radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS+) is quenched by antioxidants in the test sample. Trolox (Hoffmann-La Roche, Basel, Switzerland) was used as a standard (Blount et al., 2002). The plasma was diluted 1:30 with assay buffer (5 mmol l^{-1} potassium phosphate, pH 7.4,

containing 0.9% sodium chloride and 0.1% glucose). 10 μl of diluted samples or standards, 10 μl of metmyoglobin and 150 μl of chromogen were added per well of 96-well microplates. To initiate the reactions, 40 μl of hydrogen peroxide were added and the plate was incubated on a shaker for 5 min at room temperature. Optical density was measured at 750 nm. Results are expressed as mmol Trolox equivalent per ml of test sample. All the samples were analysed twice and the means used in the statistical analyses. Intra- and interassays coefficients of variation were 3.6 and 11.7%, respectively and repeatability of measurements was high [six repeats on seven individuals; $F_{6,35}$=134.50; P<0.0001; r=0.96 (Lessells and Boag, 1987)].

Measurements of plasma carotenoids

Total plasma carotenoids were assessed by spectrophotometry following the method of Alonso-Alvarez et al. (Alonso-Alvarez et al., 2004). Briefly, carotenoids were extracted by diluting 20 μl of plasma in 180 μl of absolute ethanol. The solution was vortexed and centrifuged for 10 min at 1500 g to precipitate the flocculent proteins. 100 μl of the supernatant were then deposited in each well of 96-well plates and the optical density was read with a microplate reader device at 450 nm. Carotenoid concentration was determined from a standard curve of lutein of known concentration (700 μg ml^{-1}). The standard curve was obtained using a serial dilution of lutein in absolute ethanol (0–20 μg ml^{-1}). Previous work has already demonstrated the reliability of this method to assess circulating carotenoids by comparison with high performance liquid chromatography (HPLC) analyses (Alonso-Alvarez et al., 2004; Eraud et al., 2007); Pearson correlation coefficient; r=0.80, P<0.0001, N=32). Intra- and interassays coefficients of variation were 7.5 and 1.7%, respectively and repeatability of measurements was high [three repeats on seven individuals; $F_{6,14}$=4652.79; P<0.0001; r=0.99 (Lessells and Boag, 1987)].

Statistical analyses

ANCOVA models were used to analyze initial values and changes in antioxidant activity, bill colour, body mass and plasma carotenoid concentration. For each of these variables we verified the normality and the homogeneity of variances. The independent variables included two factors (sex and treatment) and the age as a covariate. All the two-way interactions were also included in the model and were subsequently removed if not significant (P>0.05). Differences in sample sizes for initial values are due for reduced blood volume (one bird for plasma carotenoid concentration) and differences in samples sizes to changes throughout the experiment reflect missing values due to the death of five birds (four birds belonging to the 'immune activation' group and one bird belonging to the control group). All the statistical analyses were performed using JMP 5.0.

RESULTS
Initial values and allocation of carotenoids to coloration

Male zebra finches initially had redder bills than females [males: 6.5±0.30 (mean hue ± s.e.m.); females: 8.4±0.29], whereas body mass did not differ between sexes (Table 1). Neither bill colour nor
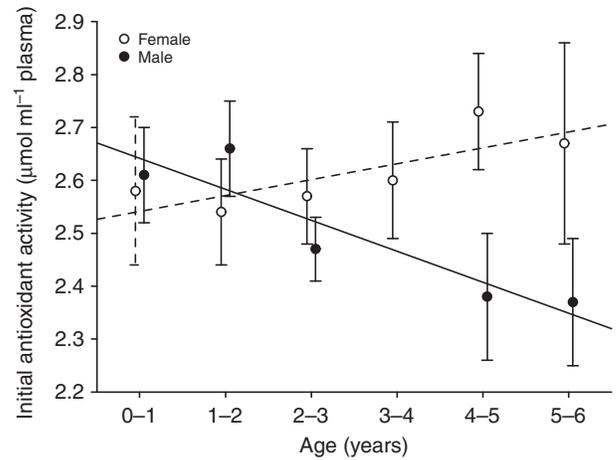

Fig. 1. Initial antioxidant activity in relation to age for male (filled circle, solid line) and female (open circle, dashed line) zebra finches. Values are mean ± s.e.m. (μmol ml^{-1} plasma).

body mass varied across ages (Table 1). Antioxidant activity depended on age in a different way in females and males (Table 1). Although older males had lower antioxidant activity than younger individuals (slope: –0.06±0.02; $F_{1,28}$=5.84; P=0.02; r^2=0.17; Fig. 1), there was no correlation between antioxidant activity and age in females ($F_{1,33}$=1.32; P=0.26; Fig. 1). Finally, circulating carotenoids did not depend on either sex or age (Table 1). To appraise the allocation of carotenoids to sexual signalling, we assessed the correlation between bill colour and circulating carotenoids for both sexes and for the different ages. Bill redness was positively correlated with the amount of circulating carotenoids ($F_{1,59}$=11.68; P=0.001). However, the strength of the correlation depended on the age of birds (carotenoids concentration × age: $F_{1,59}$=5.10; P=0.02; Fig. 2). To test this, we analysed the correlation between bill coloration and circulating carotenoids using three age classes (0–2 years old, 2–4 years old and 4–6 years old; Fig. 2). Although hue values were negatively related to the level of circulating carotenoids in young [slope: –0.10±0.03 (mean ± s.e.m.); $F_{1,21}$=11.53; P=0.003) and middle-aged birds (slope: –0.13±0.03; $F_{1,25}$=14.35; P=0.001), the correlation was no longer significant for older birds (slope: 0.07±0.07; $F_{1,13}$=1.19; P=0.30).

Changes in total antioxidant activity

Total antioxidant activity decreased throughout the experiment. Changes in antioxidant activity between day 0 and day 1 depended on both treatment and age of birds in an interactive way (Table 2, Fig. 3). Total antioxidant activity in older birds decreased more for immune-activated birds than control birds (Fig. 3), whereas the opposite pattern was observed for younger birds (Fig. 3). However, after 21 days, immune challenge and age had no effect on changes in total antioxidant activity (Table 2). Sex had no effect on changes in antioxidant activity across the experiment (all P>0.36).

Table 1. Initial values of bill colour, body mass and physiological parameters depending on sex and age of individuals

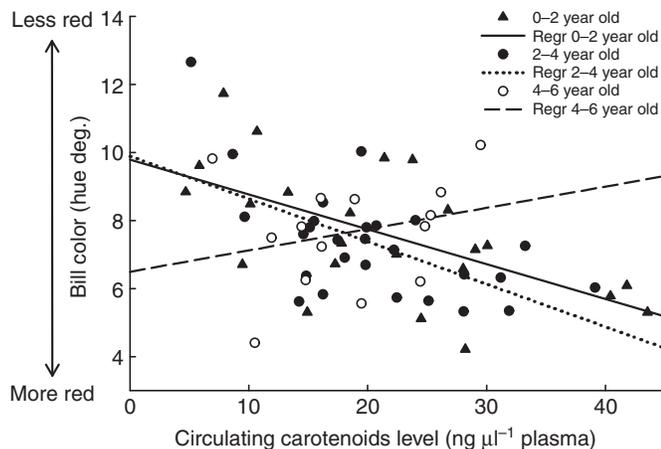|	Bill colour	Body mass	Antioxidant activity	Plasma carotenoids
Age	$F_{1,62}$=0.70 P=0.40	$F_{1,62}$=0.23 P=0.63	$F_{1,61}$=0.36 P=0.55	$F_{1,58}$=1.73 P=0.19
Sex	$F_{1,62}$=57.21 P<0.0001	$F_{1,62}$=0.01 P=0.94	$F_{1,61}$=2.29 P=0.14	$F_{1,59}$=0.003 P=0.96
Age × sex	$F_{1,61}$=0.08 P=0.77	$F_{1,61}$=0.02 P=0.90	$F_{1,61}$=5.68 P=0.02	$F_{1,58}$=0.76 P=0.39

Fig. 2. Correlation between initial bill colour and circulating carotenoids for the different age classes. Individual value in hue (deg.) in relation to age classes is shown (0–2 years old: filled triangle and solid line; 2–4 years old: filled circle and dotted line; 4–6 years old: open circle and dashed line).
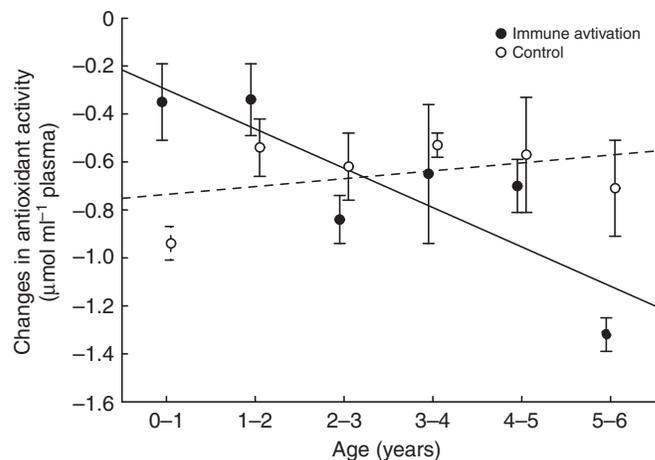

Fig. 3. Changes in antioxidant activity 24 h after the first injection depending on age for immune-activated (filled circle, solid line) and PBS-injected (open circle, dashed line) zebra finches. Mean change in antioxidant activity (μmol ml^{-1} plasma; mean ± s.e.m.) in relation to age and immune treatment is shown.

Changes in plasma carotenoid concentration

Immune activation strongly decreased levels of circulating carotenoids after 24 h ($F_{1,54}=5.81$; $P=0.02$) and also at the end of the experiment ($F_{1,54}=11.03$; $P=0.002$). However, age did not affect the way the levels of plasma carotenoids changed throughout the experiment (after 24 h: age: $F_{1,54}=0.05$, $P=0.83$; age × immune activation: $F_{1,54}=0.43$, $P=0.51$; after repeated injections: age: $F_{1,54}=0.001$, $P=0.98$; age × immune activation: $F_{1,54}=0.19$, $P=0.67$). Sex had no effect on changes in circulating carotenoids throughout the experiment (all $P>0.15$).

Changes in body mass

Birds facing an inflammatory stress lost more body mass than individuals injected with PBS during the 24 h following the injection, but this effect did not depend on their age (Table 2). After repeated injections (day 15), individual variation in body mass was affected by the immune treatment and age ($N=61$; age: $F_{1,57}=7.63$, $P=0.008$; immune activation: $F_{1,57}=7.44$, $P=0.008$; age × immune activation: $F_{1,57}=6.74$, $P=0.01$; Fig. 4). Body mass decreased with age in challenged birds, whereas it remained stable throughout the experiment for PBS-injected birds of all ages (Fig. 4). This interaction between immune activation and age was still significant 21 days post-injection (Table 2). Sex had no effect on changes in body mass across the experiment (all $P>0.2$).

Changes in bill colour

Activating the inflammatory response produced a decrease of bill redness ($F_{1,57}=5.02$, $P=0.03$). However, the effect of immune activation on bill colour changes depended on the age of birds (immune activation × age: $F_{1,57}=4.04$, $P=0.05$; age: $F_{1,57}=0.004$,

$P=0.97$; Fig. 5). The colour of challenged and control birds differed only in older individuals. To test this further, we also analysed the effect of immune activation for three age classes (0–2 years old, 2–4 years old and 4–6 years old). Bills of challenged birds became less red than those of controls only in the older individuals (Fig. 5; effect of immune treatments; 0–2 years old: $F_{1,20}=0.01$, $P=0.94$; 2–4 years old: $F_{1,23}=2.47$, $P=0.13$; 4–6 years old: $F_{1,12}=4.96$, $P=0.04$). Sex had no effect on changes in beak colour across the experiment (all $P>0.1$).

DISCUSSION
Age-related allocation of carotenoids

The free radicals theory of ageing is based on the assumption that oxidative damage accumulates over time as a result of a progressive increase in ROS/RNS production and a concomitant decline in antioxidant capacities (Finkel and Holbrook, 2000). In agreement with this view, we found that total antioxidant activity (TAA) decreased with age in male zebra finches. TAA assesses the availability of non-enzymatic antioxidants (both dietary and endogenously produced) and their capacity to scavenge ROS (Isaksson et al., 2007; Re et al., 1999). A decrease in TAA with age can be the consequence of several processes: an increase in ROS production with age, a decreased activity of enzymatic defences enhancing consumption of dietary antioxidant to sustain defences, or a weakened ability to assimilate dietary antioxidants. Since we did not assess ROS production and the activity of antioxidant enzymes, it is difficult to come to any conclusions regarding the mechanisms underlying the observed results.

Contrary to the observed decline in antioxidant defences with age, bill colour was maintained throughout the lifetime. Theory

Table 2. Changes in body mass and antioxidant activity depending on immune activation and age of individuals

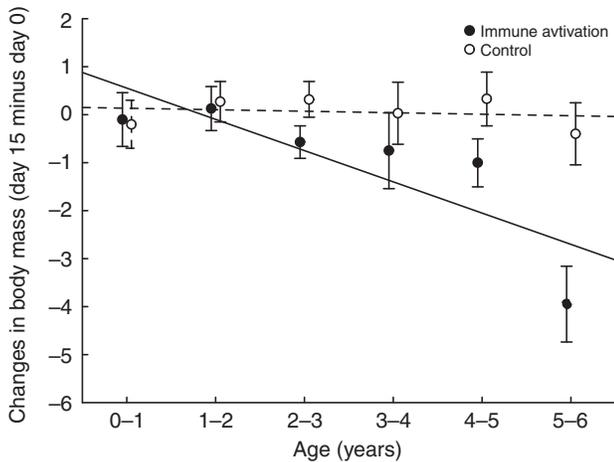| | Changes between day 0 and day 1 | | Changes between day 0 and day 21 | |
	Antioxidant activity	Body mass	Antioxidant activity	Body mass
Age	$F_{1,61}=2.82$ $P=0.098$	$F_{1,62}=0.005$ $P=0.95$	$F_{1,59}=1.57$ $P=0.21$	$F_{1,57}=3.63$ $P=0.06$
Immune treatment	$F_{1,61}=0.006$ $P=0.94$	$F_{1,63}=24.91$ $P<0.0001$	$F_{1,58}=0.27$ $P=0.60$	$F_{1,57}=0.14$ $P=0.71$
Age × immune treatment	$F_{1,61}=7.61$ $P=0.0076$	$F_{1,61}=0.39$ $P=0.53$	$F_{1,57}=0.13$ $P=0.72$	$F_{1,57}=5.00$ $P=0.03$

Fig. 4. Changes in body mass at the end of the immune treatment depending on age for immune-activated (filled circle, solid line) and PBS-injected (open circle, dashed line) zebra finches. Values are means ± s.e.m. (g) in relation to age and immune treatment.
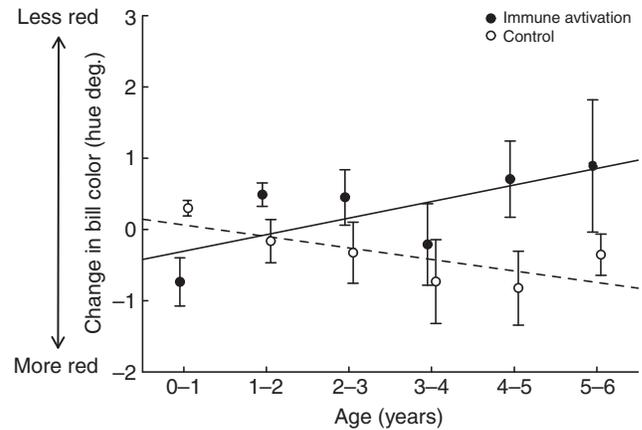

Fig. 5. Changes in bill colour during the 3 weeks of the experiment, depending on age for immune-activated (filled circle, solid line) and PBS-injected (open circle, dashed line) zebra finches. Values are mean ± s.e.m. change in bill hue (deg.) in relation to age and immune treatment.

suggests that signallers may be selected to increase signal intensity with age, as females should prefer older males as mates because their survival proves their high genotypic quality for viability (Bonduriansky et al., 2008; Kokko, 1997). Here, signal intensity did not increase with age but we observed a decline in antioxidant defences with age. This suggests that, at least in old males, individuals were more prone to allocate carotenoids to the expression of the sexual signal than to their protection against oxidative damage. In agreement with a previous study, we found that bill coloration of zebra finches was positively correlated to the amount of pigments circulating in the blood (Alonso-Alvarez et al., 2004). However, this correlation vanished in older individuals. This result suggests that old individuals allocated the same amount of carotenoids to coloration whatever the availability of circulating carotenoids. Maintaining an attractive secondary sexual trait might be adaptive for old males with a poor survival prospect. This is reminiscent of a terminal investment when old individuals allocate a large fraction of their resources to their potential last reproductive event and can divert carotenoids away from somatic maintenance in order to increase their sexual coloration in an attempt to maintain sexual attractiveness (Pike et al., 2007). Moreover, for short-lived species, fitness is more sensitive to any variation in fecundity than in adult survival, which corroborates the idea that maintaining an attractive sexual signal confers a selective advantage even when it comes at the expense of increased oxidative damage. These findings, however, raise a question about the reliability of carotenoid-based sexual traits as indicators of the antioxidant status of their bearers, at least for the oldest individuals. One might wonder whether this lack of correlation in older individuals is due to low sample sizes. However, the slopes of the correlation between bill colour and circulating carotenoids contrasted clearly between the oldest individuals and the other age classes, suggesting that the absence of such a correlation in older birds did not reflect a lack of power. Further investigation is, however, needed to elucidate if honesty of secondary sexual traits may be age dependent. Interestingly, the total antioxidant activity and the bill coloration of females were not correlated with age, suggesting that different physiological constraints might operate in the two sexes.

Immune activation and carotenoids allocation

The inflammatory response is characterized by the recruitment and activation of a number of phagocytic cells that produce highly reactive metabolites (ROS and RNS). These compounds have a cytotoxic effect that contributes to the killing of the invading pathogens. However, these molecules also participate to alter the redox balance during the inflammatory process. Several studies have shown that the availability of antioxidants (e.g. carotenoids) is generally reduced during the immune responses to infectious diseases (Allen, 1987; Augustine and Ruff, 1983; Hennet et al., 1992). This study reinforces this view, since we found that an inflammatory insult induced a significant decrease in the total antioxidant activity 24 h after the first immune challenge. Interestingly, however, this decrease was mostly due to the oldest age class, reinforcing the idea that aging individuals are more susceptible to inflammation. Although evolutionary ecologists have devoted much effort to assess the link between secondary sexual traits and maintenance functions, few studies have explicitly taken into account the age of individuals in their analyses (Alonso-Alvarez et al., 2009; Torres and Velando, 2007). Our results suggest that old individuals paid an extra cost of immune activation possibly because they experienced a stronger oxidative burst and/or are endowed with weakened antioxidant machinery. Although disentangling the two mechanisms is a difficult task, the finding that immune activation reduced the amount of circulating carotenoids independently of age, supports the view that an impairment of the endogenous antioxidant machinery might explain the observed results. Changes in body mass further confirm the extra cost of immune activation for old individuals. Indeed, after repeated immune challenge, older individuals lost even more weight than younger ones.

In addition to a decrease in antioxidant status, body mass and circulating carotenoids, activating the inflammatory response depressed the expression of a carotenoid-based sexual signal in the oldest individuals. In agreement with a previous study (Faivre et al., 2003a), our results show that immune activation directly diverted carotenoids from the sexual signal, and furthermore, that older individuals have a greater decrease of bill colour. Since ROS and RNS production increases and the enzymatic component of the

antioxidant machinery might weaken with age, the consequences of an oxidative burst triggered by the immune challenge are expected to be more detrimental in old individuals. Therefore, old individuals might invest more carotenoids to counteract the enhanced ROS/RNS production due to immune activation. In fact, the immune-induced decrease in TAA was found 24h after the first immune challenge but not after repeated immune challenges (21 days later). These results suggest that under a chronic infection, older birds can maintain their antioxidant activity over a decrease in carotenoid-dependent ornament. However, in this study, immune activation changed the amount of circulating carotenoids regardless of age, suggesting that old individuals did not use more carotenoids. Carotenoid-based sexual traits would indicate the antioxidant status of their bearers independently of carotenoid concentration in plasma. Effective antioxidant machinery can prevent the alteration and destruction of carotenoids through their oxidation. Higher antioxidant defences thus allow the organism to allocate unbleached carotenoids to coloration (Hartley and Kennedy, 2004). Additionally, our work suggests that age might be a crucial trait that should be considered for further investigation required to elucidate the carotenoid allocation process between sexual signalling and antioxidant defences. Finally, sex had no effect on age-related costs of immune activation. Although allocation strategies should differ between males and females, the overexpression of immune defences reduced antioxidant status independently of sex. Therefore, this result suggests that the inflammatory process leads to serious oxidative damage that may blur any allocation strategies differing between sexes.

Our study is based on the assumption that carotenoids are important compounds not only for the expression of a sexual signal, but also because they have the potential to alter the redox balance. The antioxidant property of carotenoids is, however, part of an ongoing debate (Costantini et al., 2007; Costantini and Moller, 2008; Hartley and Kennedy, 2004; Isaksson et al., 2007). There is a wide heterogeneity among studies that have explored the antioxidant nature of carotenoids. Summarizing these studies is obviously beyond the scope of this article. Nevertheless, we would like to emphasise the need for more research that looks at how carotenoids might act as antioxidants *in vivo*, and more importantly on how context dependency might generate the heterogeneity observed to date. The dose used in supplementation studies, the timing of supplementation, the environmental conditions, the synergistic effects among dietary and enzymatic defences and the interaction with ongoing infectious disease are some of the factors that might account for such heterogeneity.

Conclusion

Despite small sample sizes because of the reduced number of old individuals, we detected several significant effects that foster the discussion of processes that involve sexual signalling and maintenance functions. We recognise that the oldest age class only contained a handful of birds, and that these oldest birds were those that tended to drive some of the results reported here. This is of course a mere demographic consequence of time-dependent survival. There are few very old birds in a given population as there are fewer centenarians than teenagers in human populations. Our results suggest that the allocation of carotenoids to sexual signal *versus* health components may be affected by the age of individuals. Particularly, senescent males seemed to allocate more carotenoids to sexual signals than their antioxidant status allow them. This is in accordance with a terminal investment strategy in old individuals investing a large proportion of their resources to a last reproduction

event because of their poor survival prospects. A controlled inflammatory challenge confirmed the poor ability of older individuals to cope with an overproduction of free radicals as they paid an additional cost in terms of antioxidant activity, body mass and expression of carotenoid-based signals. Particularly, although the allocation of carotenoids seemed biased toward sexual signals in older individuals, an inflammatory insult altered the expression of a carotenoid-based sexual signalling in senescent individuals. The efficiency of antioxidant machinery varies with age and may explain the costliness of immune response when individuals grow old. These results may also be due to the fact that individuals that perform better die younger and thus disappear in older cohorts (i.e. 'selective disappearance'). Longitudinal data are thus needed to establish whether a selective disappearance or rather a decline in the performance explains our results. Further investigations are also required to elucidate the crucial factors that affect carotenoid allocation between secondary sexual traits and antioxidant functions. Age is probably one of these factors and deserves attention to broaden our view on signalling function of sexual ornaments. Finally, it would be worthwhile to repeat such studies using free-ranging organisms that experience the full spectrum of environmental constraints.

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