Available online at www.sciencedirect.com
ScienceDirect
Comparative Biochemistry and Physiology, Part A 145 (2006) 137–142
CBP
www.elsevier.com/locate/cbpa

Effects of T-cell-mediated immune response on avian oxidative stress

David Costantini [a,b,*], Giacomo Dell'Omo [c]

[a] *Dipartimento di Biologia Animale e dell'Uomo, Università La Sapienza, Viale dell'Università 32, 00185 Roma, Italy*
[b] *Dipartimento dell'Ambiente e Prevenzione Primaria, Unità di Chimica Tossicologica, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy*
[c] *Ornis italica, Piazza Crati 15, 00199 Roma, Italy*

Received 17 October 2005; received in revised form 5 June 2006; accepted 5 June 2006
Available online 8 June 2006

Abstract

We evaluated the oxidative cost paid by birds when coping with an immune challenge. We used the phytohaemagglutinin skin test (PHA) to assess the effects of the T-cell-mediated immune response on the concentration of reactive oxygen metabolites (ROMs), total antioxidant barrier (OXY) and total serum carotenoid concentration in wild nestlings of the Eurasian kestrel (*Falco tinnunculus*). Immunostimulation caused increased levels of ROMs, decreased OXY and increased circulating levels of carotenoids. These results suggest that an immune challenge can increase avian oxidative stress, and that carotenoids were remobilised from other tissues likely because their circulating levels were not sufficiently high to sustain an effective immune response.

Keywords: Antioxidant barrier; Carotenoids; *Falco tinnunculus*; Free radicals; Immunocompetence; Wild kestrel nestlings; PHA; ROMs

1. Introduction

Oxidative stress and its influence on avian life-history traits is a relatively new research topic, for which our knowledge is still quite limited. The balance between pro-oxidant production and antioxidant defences has important health-related implications and is considered to be the best indicator of the individual oxidative stress level (Finkel and Holbrook, 2000). In particular, oxidative damage to macromolecules is considered causally related to cellular senescence and death, and represents the main proximate mechanism driving the aging process (Harman, 1956, 1972; Beckman and Ames, 1998).

Organisms exploit a wide range of chemicals to cope with pro-oxidant compounds. Carotenoids are antioxidant pigments that have received much attention from biologists because of their ecological and evolutionary implications in vertebrate biology, particularly in birds. Indeed, it has been shown that carotenoids are correlated with some fitness traits such as egg-laying capacity (reviewed in Blount, 2004). However, they are supposed to be in limited supply for reproduction, health-related functions and the expression of sexual colouration, since they can only be acquired from dietary sources. In fact, animals are unable to synthesise them *de novo* and their availability in food is limited (Olson and Owens, 1998; Møller et al., 2000). The allocation of this limited resource among competing demands is a pivotal issue for an understanding of the role of carotenoids in the evolution of life-history traits (Blount, 2004).

The existence and importance of a trade-off in the exploitation of carotenoids for different functions, e.g. sexual advertisement and immune activation, has been suggested by different studies. Captive male blackbirds (*Turdus merula*) immunized with a suspension of sheep red blood cells displayed a significant decrease in bill colour, suggesting that activation of the immune system removes carotenoids stored in the beak in order to enhance the immune response (Faivre et al., 2003). In fact, carotenoids play an important role in the modulation of avian immune functions: individuals with higher circulating carotenoid levels produce larger T-cell-mediated and humoral immune responses (Blount et al., 2003b; McGraw and Ardia, 2003). In addition, the recruitment of carotenoids from tissues

* Corresponding author. Dipartimento di Biologia Animale e dell'Uomo, Università La Sapienza, Viale dell'Università 32, 00185 Roma, Italy. Tel.: +39 0649914780; fax: +39 064958259.
E-mail address: david.costantini@uniroma1.it (D. Costantini).

1095-6433/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpa.2006.06.002

might be important to cope with free radical production during the immune response. The cytotoxic effects of free radicals produced by leukocytes are exploited by the organism when coping with pathogens during the inflammatory response (Klebanoff and Clark, 1978; von Schantz et al., 1999). An excessive production of free radicals can increase the level of oxidative stress if not effectively counteracted by the organism's antioxidant defences. In this regard, carotenoids might also be recruited from tissues to help limit the oxidative damage. To our knowledge, however, the simultaneous effects of immunostimulation on pro-oxidant compounds, total antioxidant barrier and serum carotenoids have never been investigated.

In this study, we measured the effects of the T-cell-mediated immune response elicited by the phytohaemagglutinin skin test (PHA) on the concentration of reactive oxygen metabolites (ROMs), total serum antioxidant barrier (OXY) and total serum carotenoid concentration in wild nestlings of the Eurasian kestrel (*Falco tinnunculus*), a raptor which displays carotenoid-based colouration of the skin (Casagrande et al., 2006). In this species, the relationship between ROMs and OXY and their correlates have been recently studied (Costantini et al., 2006; Costantini and Dell'Omo, in press). Our aims were to evaluate i) whether the immune challenge via mitogen action could lead to an oxidative cost in terms of an imbalance toward pro-oxidant compounds, and ii) the response of circulating carotenoids.

2. Materials and methods

2.1. Field work

The field work was carried out during the 2005 breeding season in a 1200 km^2 area around Rome. We collected data from nest-boxes mounted on the utility lines of two local electric power companies. Nests were distributed in cultivated (i.e. cereal fields) and uncultivated areas (i.e. fallow fields and pastures). Thirty-one 24–26-day-old nestlings from seven nests were used for the experiment. There were five nests with four chicks, one with five, and one with six nestlings. Hatching dates were assessed by repeated visits to the nests in the perihatching period. Within each brood (except the five-chick one), half of the nestlings were randomly assigned to two treatment groups, i.e. controls or immunostimulated. In the five-chick brood, two specimens were assigned to the controls and three to the immunostimulated. A blood sample (400 μL) was drawn from the brachial vein just before injection (time 0) and 24 h later (time 1). The samples were kept cool (0–5 °C) until centrifugation, which occurred within a few hours, and the serum was stored at −20 °C.

2.2. Phytohaemagglutinin test

A phytohaemagglutinin assay (PHA) was used to evaluate the *in vivo* effects of the T-cell-mediated immune response (CMI) on ROMs, OXY and carotenoid concentration. PHA is a mitogen commonly used in bird studies to evaluate thymus-dependent functions (Goto et al., 1978; Merino et al., 1999). Nestlings were injected intradermically in the wing patagium with 50 μL of 1 mg mL^{-1} PHA (Sigma-Aldrich) dissolved in phosphate-buffered saline (PBS), while control individuals were injected with 50 μL of PBS alone, according to Smits et al. (1999). The skin was swabbed with alcohol just prior to injection.

The thickness of the wing web was measured three times with a calliper (to the nearest 0.01 mm) at the injection site just before (time 0) and 24 h after injection (time 1). As the repeatabilities (Lessells and Boag, 1987) of both initial ($r=0.978$, $F_{30,62}=136.58$, $p<0.001$) and final ($r=0.995$, $F_{30,62}=601.09$, $p<0.001$) measurements were high, the mean values were used for statistical analyses.

2.3. Measurement of reactive oxygen metabolites

Reactive oxygen species (ROS) are extremely reactive with macromolecules such as lipids, proteins, and nucleic acids and form derivatives that maintain oxidizing properties such as reactive oxygen metabolites (ROMs). These compounds are able to propagate the oxidation cascade. The serum concentration of ROMs (primarily hydroperoxides, ROOH) was measured by the d-ROMs test (Diacron, Grosseto, Italy). The serum (20 μL) was first diluted with 200 μL of a solution containing 0.01 M acetic acid/sodium acetate buffer (pH 4.8) and *N*,*N*-diethyl-*p*-phenylenediamine as chromogen and then incubated for 75 min at 37 °C. The acidic pH favours the release of iron (Fe^{2+} and Fe^{3+}) and copper (Cu^{+} and Cu^{2+}) from serum proteins. These metals catalyse the cleavage of ROOH, leading to the generation of two highly reactive and histolesive pro-oxidants, namely the alkoxyl (R-O˙) and alkylperoxyl (R-OO˙) radicals. When these compounds react with an alkyl-substituted aromatic amine (A-NH_2) solubilized in the chromogen, they produce a complex whose colour intensity (pink) is directly proportional to their concentration. After incubation, the absorbance was read with a spectrophotometer (Microplate Reader Model 550) at 490 nm and the concentration of ROMs was calculated by comparison with a standard curve obtained by measuring the absorbance of a standard solution. The results of the d-ROMs test are expressed in arbitrary units called "Carratelli units" (CARR U), where 1 CARR U corresponds to 0.08 mg of H_2O_2/100 mL. The standard solution (after adequate dilutions) was equivalent to a H_2O_2 concentration ranging from 0.18 to 5.88 mM. The ROMs were expressed as mM of H_2O_2. Previous tests of repeatability showed a high reliability of the method (pooled sample repeatability: $r=0.964$, $p<0.001$; intraplate repeatability: range 0.696 to 0.956, all $p<0.001$; see Lessells and Boag, 1987).

2.4. Measurement of antioxidant barrier

A suite of enzymatic and non-enzymatic antioxidant defences are induced by the increase in pro-oxidant concentration in order to counteract and regulate overall pro-oxidant levels and to maintain redox homeostasis (e.g. Halliwell and Gutteridge, 1989, 1990). The serum antioxidant barrier (OXY)

includes both exogenous (e.g. ascorbate, tocopherols, carotenoids) and endogenous (e.g. uric acid, enzymes) compounds. The total antioxidant barrier was measured by the OXY-Adsorbent test (Diacron, Grosseto, Italy). This dedicated kit uses a colorimetric determination to quantify the ability of the antioxidant barrier to cope with the oxidant action of hypochlorous acid (HOCl; pro-oxidant of pathologic relevance in biological systems). The serum (10 μL) was diluted 1:100 with distilled water. A 200 μL aliquot of a titred HOCl solution was incubated with 5 μL of the diluted serum for 10 min at 37 °C. Then, 5 μL of the same chromogen solution used for the ROMs determination was added. An alkyl-substituted aromatic amine solubilized in the chromogen is oxidized by the residual HOCl and transformed into a pink derivative. The intensity of the coloured complex, which is inversely related to the antioxidant power, was measured with the same spectrophotometer at 490 nm. Calibration was achieved by means of a human serum able to neutralise 440 μmol HOCl mL^{-1}. The standard curve (range: 0.275–8.8 μmol mL^{-1}; all values are subsequently multiplied ×100 to correct for dilution) was determined using the same spectrophotometer. Measurements were expressed as mM of HOCl neutralised. Previous tests of repeatability showed a high reliability of the method (pooled sample repeatability: $r=0.875$, $p<0.001$; intraplate repeatability: range 0.529 to 0.647, all $p<0.001$; see Lessells and Boag, 1987).

2.5. Measurement of carotenoids

The serum (100 μL) was diluted with absolute methanol (1:8) and the flocculent proteins were precipitated by centrifugation at 12000×*g* for 5 min. Carotenoids were quantified by means of a Beckman DU 7400 spectrophotometer at 476 nm. The carotenoid concentration was estimated as micrograms per milliliter of serum using the standard absorbance curve of lutein (alpha-carotene-3,3′-diol; Sigma-Aldrich), which represents about 90% of the total xanthophyll content in both blood and skin of the Eurasian kestrel (zeaxanthin is the only other carotenoid present; Casagrande et al., 2006).

2.6. Statistical analyses

ANOVA for repeated measures (RM) was used to compare the measurements before (time 0) and 24 h after the treatment (time 1) in order to detect the effects of the immunostimulation on the swelling of the wing web, ROMs, OXY and serum carotenoid concentration. Treatment was included as fixed factor and Nest as random factor to avoid pseudoreplication (Hurlbert, 1984). All the statistical analyses were performed with STATISTICA 6.0 (StatSoft 2001, Tulsa, USA).

3. Results

No significant differences in body mass were detected between the two treatment groups before or after the test ($F_{1,6}=0.13$, $p=0.73$; Treatment × RM: $F_{1,6}=0.02$, $p=0.89$). As expected, the immunostimulation caused a four-fold swelling of the wing web in the treated nestlings compared to controls and to the pre-treatment measurements (Treatment × RM: $F_{1,6}=76.56$, $p<0.001$; post-hoc comparisons with the Tukey test: all $p<0.001$). None of the other interactions were significant.

The concentration of ROMs increased in immunostimulated nestlings (Treatment × RM: $F_{1,6}=6.01$, $p=0.049$; Fig. 1a). Conversely, immunostimulation resulted in a significant decrease of the antioxidant barrier (Treatment × RM: $F_{1,6}=14.34$, $p=0.009$; Fig. 1b). No main effects or interaction of the other factors on these two variables emerged from the analyses.

A significant inter-nest variation in carotenoid concentration was found (Nest: $F_{6,17}=4.11$, $p=0.01$; Nest × RM: $F_{6,17}=2.59$, $p=0.058$). However, the carotenoid concentration increased in immunostimulated nestlings (Treatment × RM: $F_{1,6}=6.49$,

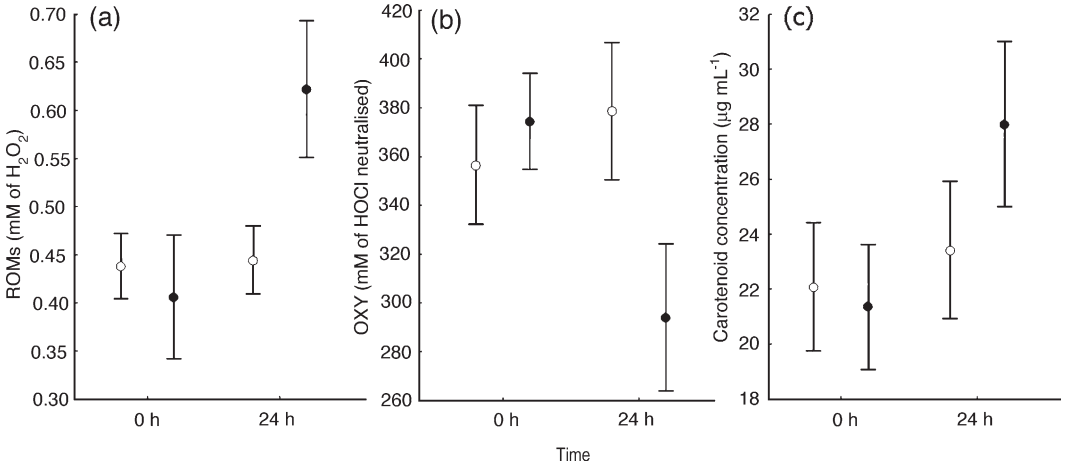

Fig. 1. Effects of T-cell-mediated immune response caused by an intradermal phytohaemagglutinin injection (PHA) on serum reactive oxygen metabolite (ROMs) concentration (a), serum antioxidant barrier (OXY) (b), and serum carotenoid concentration (c) in wild kestrel nestlings (*Falco tinnunculus*). The increase of the serum concentration of both ROMs ($p=0.049$) and carotenoids ($p=0.044$) and the depletion of the antioxidant barrier ($p=0.009$) were all significant. Data are shown as mean ±S.E. (○ controls, $n=15$; ● immunostimulated, $n=16$).

$p=0.044$; Fig. 1c). None of the other interactions were significant.

4. Discussion

Our study is the first to measure the effects of immunostimulation on oxidative stress in wild birds. The results showed that Eurasian kestrel nestlings responded to immunostimulation with an increase in pro-oxidant concentration and a depletion of the total antioxidant defences. The imbalance between these two variables is interpreted as an increase of the oxidative stress level (e.g. Finkel and Holbrook, 2000).

Most of the endogenous pro-oxidant production is accounted for by different sources such as the mitochondrial electron transport and the immune response (e.g. Ames et al., 1993). The PHA method induces the infiltration of T-lymphocytes and macrophages at the site of the injection, resulting in a local and not a systemic response to an immune insult. However, ROMs represent an oxidative challenge for the whole organism. The toxicity of these metabolites is promoted by the presence of metals which catalyse their cleavage, leading to the generation of two highly reactive and histolesive pro-oxidants, namely the alkoxyl (R-O·) and alkylperoxyl (R-OO·) radicals (e.g. Halliwell and Gutteridge, 1989; Leffler, 1993; Iorio, 2004). The concomitant increase in ROMs concentration and decrease of the antioxidant barrier suggests that the antioxidant system of the nestlings could not effectively cope with a sudden overproduction of pro-oxidant compounds. Conversely, in a recent study, immunostimulation by intraperitoneal injection with lipopolysaccharide of *Escherichia coli* in adult zebra finches (*Taenyopigia guttata*) did not impair their antioxidant power (Alonso-Alvarez et al., 2004). In our case, the high metabolic rate of nestlings during growth (Vleck and Bucher, 1998) combined with their incomplete antioxidant machinery (e.g. Blount et al., 2003a) could partly explain the poorer ability of the Eurasian kestrel chicks than of the adult zebra finches to cope with oxidative stress.

The leukocytes activated during the inflammatory response release pro-oxidant compounds which have a damaging effect on pathogens. For example, the generation of free radicals during the cellular immune response elicited by a coccidial (*Eimeria maxima*) infection in chickens (*Gallus gallus*) caused a reduction of plasma carotenoids (Allen, 1997). This reduction was related to the generation of reactive oxygen intermediates rather than to their malabsorption (Allen et al., 1996; Allen, 1997). Similarly, a decrease in plasma carotenoids occurred after immunostimulation by *E. coli* in chickens (Koutsos et al., 2003a) and zebra finches (*T. guttata*, Alonso-Alvarez et al., 2004), as well as after a PHA and a sheep red blood cell haemagglutinin (SRBC) assay in both control and carotenoid-supplemented male zebra finches (McGraw and Ardia, 2003).

In contrast to the above-mentioned results, the nestlings in our study responded to immunostimulation by increasing their level of circulating carotenoids. Since they could not achieve the levels we measured in 1 day from food (data in preparation), it is likely that the rise in serum concentration was due to remobilisation from skin, fat reserves and liver. This also suggests that the physiological circulating levels of carotenoids at the time of the treatment were not sufficiently high to sustain an effective immune response. This is in line with recent studies showing that carotenoid-supplemented zebra finches had significantly better immune responses than controls (Blount et al., 2003b; McGraw and Ardia, 2003). The relationship between carotenoids and the immune system has been found in different studies. For example, it has been shown in growing chicks (*G. gallus*) a carotenoid deposition into the bursa of Fabricius (Koutsos et al., 2003b), where a substantial portion of avian B-cell development occurs. Moreover, it has been found a marked stimulatory action of carotenoids on the growth of the thymus gland and on various components of the immune system in various vertebrate species (see review in Chew and Park, 2004).

The difference between our study and the others may be partly related to the magnitude and duration of the immune challenge, as well as to differences in the timing of measurements. There may also be two other explanations. One could be that the levels of carotenoids stored in the organism are species-specific, as well as being related to the age of the individual. The other is that on average, carnivores such as the kestrel obtain less carotenoids per unit mass of food than herbivores or omnivores (Olson and Owens, 1998). It has been shown that both the diet and the phylogeny explain most of the interspecific variation of circulating carotenoids in free-living and captive birds (Tella et al., 2004; McGraw, 2005). Therefore, it could be that carnivorous birds have evolved antioxidant mechanisms that rely less on carotenoids than those of granivorous (or non-carnivorous) species. Recently, it has been shown that carotenoid concentration in wild nestlings of the Eurasian kestrel (*F. tinnunculus*) is not correlated with the individual oxidative stress (Costantini et al., 2006). The fact that antioxidant defences are not uniform has been incorporated into the free radical theory, and differences in antioxidant defences have been invoked to explain differences in life span (Beckman and Ames, 1998). Finally, it should be stressed that our study was carried out in the wild and not in the laboratory.

The concomitant increase of the carotenoid concentration and depletion of the antioxidant system in nestlings suggests two possible interpretations of the results. Avian species displaying carotenoid-based colours are supposed to convey honestly, via the intensity of the colour, their ability to cope with microbial and macroparasitic infections and with oxidative stress (Lozano, 1994; von Schantz et al., 1999). In particular, the higher the effectiveness of the (non-carotenoid) antioxidant defences against free radicals the higher the likelihood that carotenoids are preserved in the skin for the production of skin colour (von Schantz et al., 1999; Hartley and Kennedy, 2004). We could not measure the magnitude of the skin colour changes because the 24-h period was too short to reveal any significant variation and the kestrels fledged just after the end of the experiment. Nevertheless, our results suggest that even if carotenoids are remobilised from sinks to mount a detoxifying response, their contribution to the antioxidant system is likely

secondary to that of other compounds, as indicated by the large decrease in the antioxidant barrier. According to studies showing that the enzymatic activity is primarily intracellular (humans in Halliwell and Gutteridge, 1990; birds in Lin et al., 2004a,b), our results suggest that other antioxidants such as vitamins and uric acid (Lin et al., 2004a,b) could be the most important chemicals used to cope with pro-oxidants in the avian circulation system. Among the many compounds with anti-oxidant properties, vitamin C has been suggested to be part of a "first line of defence", whereas lipid soluble compounds such as carotenoids may be linked mainly to lipid peroxidation (Dotan et al., 2004). Furthermore, tocopherols are known to be more efficient at trapping free radicals than carotenoids (Palozza and Krinsky, 1992). It should be noted that carotenoids can also protect against oxidative stress primarily by modulating the levels of other antioxidants, and only secondarily providing a direct antioxidant activity (Mayne and Parker, 1989; Ewen et al., 2006).

Our results suggest that birds face a trade-off when shunting carotenoids to immune function and colouration (Lozano, 1994). The trade-off between immune activation and sexual signalling is generated by the fact that mounting an immune response uses up carotenoids that are no longer available for the production of the sexual signal. Carotenoids are considered a limited resource (in food), and therefore in limited supply for the many functions in which they are involved (Blount, 2004). The carotenoid content varies among food types (Goodwin, 1984) and dietary differences have been shown to explain the interpopulation variation of blood carotenoids in different animal groups (e.g. birds in Negro et al., 2000; reptiles in Costantini et al., 2005b). Kestrels can adopt different feeding styles and hence acquire different amounts of carotenoids even if hunting in the same area (Costantini et al., 2005a).

Finally, it has been suggested that the conditions experienced during the perinatal period can impact on many phenotypic traits later in life (Lindström, 1999; Metcalfe and Monaghan, 2001). In our study, the activation of T-lymphocytes as a response to an injected mitogen showed that birds faced with an infection (e.g. parasites) can pay a physiological cost in terms of increased oxidative damage if they cannot cope with it via an effective antioxidant system. It is known in mammals that chronic infection by viruses or parasites results in a chronic phagocytic activity and consequent chronic inflammation, which cause damage to macromolecules and mutation (reviewed in Ames et al., 1993). Moreover, it has been demonstrated that free radicals can cause immunosuppressive effects through the damaging of the immune cells (Huang et al., 1992). Therefore, it is likely that birds coping with a more prolonged insult could suffer a higher degree of oxidative stress that could lower their fitness.

This study presents novel results on the relationship between immune activation, oxidative stress, and carotenoids in free-living birds. Future studies should deepen this topic by taking into account also the humoral immune response. This can help to better understand the effect of oxidative stress on life-history traits, as well as the mechanisms underlying the pattern and tempo of senescence in vertebrates.

Acknowledgements

Gianfranco Brambilla, Alberto Fanfani and the association *Ornis italica* for their support of this research; Peter Christie for the revision of the English; two anonymous reviewers for valuable comments that improved the manuscript. David Costantini was supported by a PhD fellowship from the University *La Sapienza*.

References

Allen, P.C., 1997. Production of free radical species during *Eimeria maxima* infections in chickens. Poult. Sci. 76, 814–821.

Allen, P.C., Danforth, H.D., Morris, V.C., Levander, O.A., 1996. Association of lowered plasma carotenoids with protection against cecal coccidiosis by diets high in n-3 fatty acids. Poult. Sci. 75, 966–972.

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Gaillard, M., Prost, J., Faivre, B., Sorci, G., 2004. An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. Am. Nat. 164, 651–659.

Ames, B.N., Shigenaga, M.K., Hagen, T.M., 1993. Oxidants, antioxidants, and the degenerative diseases of aging. Proc. Natl. Acad. Sci. U. S. A. 90, 7915–7922.

Beckman, K.B., Ames, B.N., 1998. The free radical theory of aging matures. Physiol. Rev. 78, 547–581.

Blount, J.D., 2004. Carotenoids and life-history evolution in animals. Arch. Biochem. Biophys. 430, 10–15.

Blount, J.D., Metcalfe, N.B., Arnold, K.E., Surai, P.F., Devevey, G.L., Monaghan, P., 2003a. Neonatal nutrition, adult antioxidant defences and sexual attractiveness in the zebra finches. Proc. R. Soc. Lond., B 270, 1691–1696.

Blount, J.D., Metcalfe, N.B., Birkhead, T.R., Surai, P.F., 2003b. Carotenoid modulation of immune function and sexual attractiveness in zebra finches. Science 300, 125–127.

Casagrande, S., Csermely, D., Pini, E., Bertacche, V., Tagliavini, J., 2006. Skin carotenoid concentration correlates with male hunting skill and territory quality in the kestrel (*Falco tinnunculus*). J. Avian Biol. 37, 190–196.

Chew, B.P., Park, J.S., 2004. Carotenoid action on the immune response. J. Nutr. 134, 257S–261S.

Costantini, D., Dell'Omo, G., in press. Environmental and genetic components of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). J. Comp. Physiol. B.

Costantini, D., Casagrande, S., Di Lieto, G., Fanfani, A., Dell'Omo, G., 2005a. Consistent differences in feeding habits between neighbouring breeding kestrels. Behaviour 142, 1409–1421.

Costantini, D., Dell'Omo, G., Casagrande, S., Fabiani, A., Carosi, M., Bertacche, V., Marquez, C., Snell, H., Snell, H., Tapia, W., Gentile, G., 2005b. Inter-population variation of carotenoids in Galápagos land iguanas (*Conolophus subcristatus*). Comp. Biochem. Physiol. B 142, 239–244.

Costantini, D., Casagrande, S., De Filippis, S., Brambilla, G., Fanfani, A., Tagliavini, J., Dell'Omo, G., 2006. Correlates of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). J. Comp. Physiol. B 176, 329–337.

Dotan, Y., Lichtenberg, D., Pinchuk, I., 2004. Lipid peroxidation cannot be used as a universal criterion of oxidative stress. Prog. Lipid Res. 43, 200–227.

Ewen, J.G., Thorogood, R., Karadas, F., Pappas, A.C., Surai, P.F., 2006. Influences of carotenoid supplementation on the integrated antioxidant system of a free living endangered passerine, the hihi (*Notiomystis cincta*). Comp. Biochem. Physiol. A 143, 149–154.

Faivre, B., Grégoire, A., Préault, M., Cézilly, F., Sorci, G., 2003. Immune activation rapidly mirrored in a secondary sexual trait. Science 300, 103.

Finkel, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of ageing. Nature 408, 239–247.

Goodwin, T.W., 1984. The biochemistry of carotenoids. Animals, vol. II. Chapman and Hall, London.

Goto, N., Kodama, H., Okada, K., Fujimoto, Y., 1978. Suppression of phytohemagglutinin skin response in thymectomized chickens. Poult. Sci. 57, 246–250.

Halliwell, B.H., Gutteridge, J.M.C., 1989. Free Radicals in Biology and Medicine. Oxford University Press, Oxford.

Halliwell, B.H., Gutteridge, M.C., 1990. The antioxidants of human extracellular fluids. Arch. Biochem. Biophys. 280, 1–8.

Harman, D., 1956. Aging: a theory based on free radical and radiation chemistry. J. Gerontol. 11, 298–300.

Harman, D., 1972. The biological clock: the mitochondria? J. Am. Geriatr. Soc. 20, 145–147.

Hartley, R.C., Kennedy, M.W., 2004. Are carotenoids a red herring in sexual display? Trends Ecol. Evol. 19, 353–354.

Huang, H.C., Chang, J.H., Tung, S.F., Wu, R.T., Foegh, M.L., Chu, S.H., 1992. Immunosuppressive effects of emodin, a free radical generator. Eur. J. Pharmacol. 211, 359–364.

Hurlbert, S.H., 1984. Pseudoreplication and the design of ecological field experiments. Ecol. Monogr. 54, 187–211.

Iorio, E.L., 2004. Il d-ROMs test e la valutazione globale dello stress ossidativo. Diacron International, Pistoia.

Klebanoff, S.J., Clark, R.A., 1978. The Neutrophil: Function and Clinical Disorders. North-Holland, Amsterdam.

Koutsos, E.A., Calvert, C.C., Klasing, K.C., 2003a. The effect of an acute phase response on tissue carotenoid levels of growing chickens (*Gallus gallus domesticus*). Comp. Biochem. Physiol. A 135, 635–646.

Koutsos, E.A., Clifford, A.J., Christopher Calvert, C., Klasing, K.C., 2003b. Maternal carotenoid status modifies the incorporation of dietary carotenoids into immune tissues of growing chickens (*Gallus gallus domesticus*). J. Nutr. 133, 1132–1138.

Leffler, J.E., 1993. An Introduction to Free Radicals. John Wiley and Sons, New York.

Lessells, C.M., Boag, P.T., 1987. Unrepeatable repeatabilities: a common mistake. Auk 104, 116–121.

Lin, H., Decuypere, E., Buyse, J., 2004a. Oxidative stress induced by corticosterone administration in broiler chickens (*Gallus gallus domesticus*): 1. Chronic exposure. Comp. Biochem. Physiol. B 139, 737–744.

Lin, H., Decuypere, E., Buyse, J., 2004b. Oxidative stress induced by corticosterone administration in broiler chickens (*Gallus gallus domesticus*): 2. Short-term effect. Comp. Biochem. Physiol. B 139, 745–751.

Lindström, J., 1999. Early development and fitness in birds and mammals. Trends Ecol. Evol. 14, 343–348.

Lozano, G.A., 1994. Carotenoids, parasites, and sexual selection. Oikos 70, 309–311.

Mayne, S.T., Parker, R.S., 1989. Antioxidant activity of dietary canthaxanthin. Nutr. Cancer 12, 225–236.

McGraw, K.J., 2005. Interspecific variation in dietary carotenoid assimilation in birds: links to phylogeny and color ornamentation. Comp. Biochem. Physiol. B 142, 245–250.

McGraw, K.J., Ardia, D.R., 2003. Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. Am. Nat. 162, 704–712.

Merino, S., Martinez, J., Møller, A.P., Sanabria, L., de Lope, F., Perez, J., Rodriguez-Caabeiro, F., 1999. Phytohaemagglutinin injection assay and physiological stress in nestling house martins. Anim. Behav. 58, 219–222.

Metcalfe, N.B., Monaghan, P., 2001. Compensation for a bad start: grow now, pay later? Trends Ecol. Evol. 16, 254–260.

Møller, A.P., Biard, C., Blount, J.D., Houston, D.C., Ninni, P., Saino, N., Surai, P.F., 2000. Carotenoid-dependent signals: indicators of foraging efficiency, immunocompetence or detoxification ability? Avian Poult. Biol. Rev. 11, 137–159.

Negro, J.J., Tella, J.L., Blanco, G., Forero, M.G., Garrido-Fernández, J., 2000. Diet explains interpopulation variation of plasma carotenoids and skin pigmentation in nestling white storks. Physiol. Biochem. Zool. 73, 97–101.

Olson, V.A., Owens, I.P.F., 1998. Costly sexual signals: are carotenoids rare, risky or required? Trends Ecol. Evol. 13, 510–514.

Palozza, P., Krinsky, N.I., 1992. Antioxidant effects of carotenoids *in vivo* and *in vitro*: an overview. Methods Enzymol. 213, 403–419.

Smits, J.E., Bortolotti, G.R., Tella, J.L., 1999. Simplifyng the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence. Funct. Ecol. 13, 567–572.

Tella, J.L., Figuerola, J., Negro, J.J., Blanco, G., Rodríguez-Estrella, R., Forero, M.G., Blázquez, M.C., Green, A.J., Hiraldo, F., 2004. Ecological, morphological and phylogenetic correlates of interspecific variation in plasma carotenoid concentration in birds. J. Evol. Biol. 17, 156–164.

Vleck, C.M., Bucher, T.L., 1998. Energy metabolism, gas exchange, and ventilation. In: Starck, J.M., Ricklefs, R.E. (Eds.), Avian Growth and Development: Evolution Within the Altricial–Precocial Spectrum. Oxford University Press, Oxford, pp. 89–116.

von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D., Wittzell, H., 1999. Good genes, oxidative stress and condition-dependent sexual signals. Proc. R. Soc. Lond. B 266, 1–12.