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Carotenoids in evolutionary ecology: re-evaluating the antioxidant role

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The antioxidant role of carotenoids in the living organism was proposed as a possible basis for the honesty of carotenoid-based signals. However, recent studies have questioned the relevance of carotenoids as powerful antioxidants *in vivo*. Current evidence does not seem to support the ''antioxidant role'' hypothesis, but it does not allow us to reject it either. This paper proposes some steps to solve this controversy, such as taking a dynamic approach to antioxidant responses, designing protocols that expose individuals to oxidative challenges, analyzing tissues other than blood, and obtaining measures of antioxidant capacity and oxidative damage simultaneously. However, it should be considered that, irrespective of their antioxidant potential, carotenoids might still give information on oxidative stress levels if they are particularly sensitive to free radicals. Finally, lumping together the immunostimulatory and antioxidant roles of carotenoids should be avoided as these functions are not necessarily associated.

Keywords: honest signaling; immune response; ornaments; oxidative stress; reactive oxigen species

Introduction

The study of carotenoid-based ornaments as honest signals of individual quality has been one of the most relevant topics in behavioral and evolutionary ecology over the last two decades.[1,2] Given that carotenoid pigments cannot be synthesized *de novo* by animals, and must be obtained through their diet, pioneer papers suggested that carotenoid-based ornaments could act as honest signals of foraging capacity and overall body condition (the ''foraging ability'' hypothesis) (Fig. 1).[3–5] However, the publication of the seminal paper of Lozano[6] offered an alternative hypothesis, suggesting that carotenoids could be involved in immune response and parasite resistance. Later, von Schantz et al.[7] incorporated these ideas into a broader context, highlighting the connections between oxidative stress and immunity and paying attention to the antioxidant properties of carotenoids (as mentioned by Lozano).[6] This resulted in an alternative honesty in carotenoid-based signals (the ''antioxidant role'' hypothesis) that relies on the assumption that carotenoid pigments have physiological functions other than pigmentiation of skin, feathers or scales. Specifically, this hypothesis assumes that carotenoids are relevant antioxidants in the body, which links it to immunostimulantion. Thus, individuals potentially face a trade-off between allocating available carotenoids for self-maintenance functions and ornamental coloration. Healthier individuals would require lower amounts of carotenoids for antioxidant functions, allowing instead allocation of these pigments to ornament expression, subsequently signaling their quality to conspecifics (Fig. 1).

Initial reviews[1,2] supported the viability of the ''antioxidant role'' hypothesis and, over the last 15 years, it has generated considerable interest among ecologists as a compelling and exciting (although not mutually exclusive) alternative to the ''foraging ability'' hypothesis. However, recent studies have questioned the key assumption of this ''antioxidant role'' hypothesis, claiming that carotenoids are not as powerful antioxidants as initially proposed.[8,9] For instance, it has been suggested that carotenoids are poor antioxidants but are particularly sensitive to be altered by reactive oxigen species (ROS), which may result in a spurious relationship between carotenoid pigmentation and oxidative stress that is mediated by the quality of the antioxidant system.[8] In addition, a recent review suggests that circulating carotenoids do not contribute much to the antioxidant defenses of birds.[9] Therefore, despite the disproportionate attention paid by behavioral ecologists to carotenoids as signals of oxidative stress and health, it seems that the crucial assumption of this hypothesis deserves further evaluation.

In this article I analyze: (i) the evidence supporting the hypothetical antioxidant role of carotenoids; (ii) the reasons that may have led to the current controversy in this topic, and (iii) the steps necessary to clarify this issue. As most studies on this topic have used birds as study models, this paper is slightly biased to this taxonomic group. However, most of the conclusions and future prospects are applicable to other taxa.

Carotenoids and ROS

Carotenoids are large lipophilic molecules composed of a chain of 40 carbon atoms joined with alternating single and

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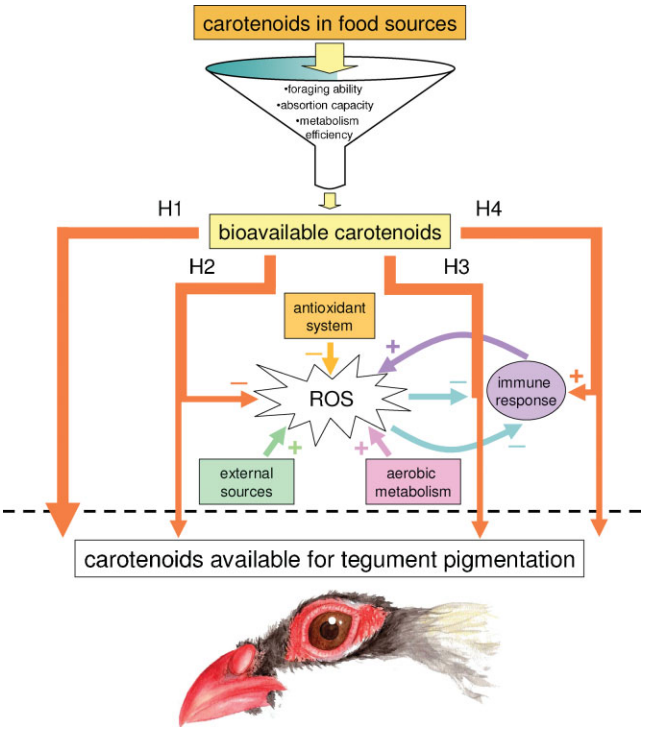

Figure 1. Schematic representation of the trade-offs and physiological pathways proposed by the four main hypothesis (H1–H4) aimed at explaining the honesty of carotenoid-based traits. The amount of carotenoids available for physiological functions in the organism (bioavailable carotenoids) is mostly limited by the foraging ability and the capacity of the individual to absorb, transport and metabolize ingested carotenoids efficiently. According to the "foraging ability" hypothesis (H1), the primary role of bioavailable carotenoids is tegument pigmentation, resulting in trait coloration conveying information about the capacity of the individual to acquire and process carotenoids. The "antioxidant role" hypothesis (H2) assumes that carotenoids are relevant antioxidants in the body, so individuals face a trade-off between allocating bioavailable carotenoids to ROS quenching *versus* tegument pigmentation. ROS are produced as a result of aerobic metabolism, immune system activation or various external factors (*e.g.*, polution, UV radiation, *etc.*), and are counteracted by the antioxidant system (that, after the "antioxidant role" hypothesis, includes the participation of carotenoids). The higher the ROS levels, the higher is the proportion of bioavailable carotenoids allocated to ROS quenching. This results in lower amounts of carotenoids being allocated to trait pigmentation, which results in tegument coloration mirroring the oxidative stress level of the individual. However, carotenoid-based traits may also reveal oxidative stress if carotenoids are not significant antioxidants, but are, in turn, particularly prone to be bleached by ROS (H3). Finally, carotenoids may play a immunostimulatory role in the organism. This would lead to a trade-off between allocating bioavailable carotenoids to immune response *versus* tegument pigmentation (H4), resulting in carotenoid-based traits conveying information about the health status of the individual.

double bonds. In most carotenoids, this linear hydrocarbon skeleton is cyclized at both ends, and these end-rings are often substituted by different functional groups. There are about 700 carotenoids in living organisms, although only a limited subset of them are found in significant amounts in animals.[10] This diverse array of carotenoids can be divided into two major groups according to their functional groups: Firstly, carotenes (*e.g.*, α-carotene, β-carotene, lycopene), which contain only carbon and hydrogen, and secondly. xanthophylls, which contain at least one oxygen atom. In addition, xanthophylls can be subdivided into hydroxy-carotenoids (*e.g.*, lutein, zeaxanthin) or ketocarotenoids (*e.g.*, asthaxanthin, canthaxanthin), depending on whether the oxygen-containing substituent is a hydroxyl or a ketone, respectively. Although diet ultimately constrains the amount and type of carotenoid in an animal, it should be noted that animals are able to perform some metabolic transformations of ingested carotenoids, so the array of specific carotenoids present in the organism may differ from that ingested.

The color of carotenoid-based traits (crucial in their role as honest signals of quality) is determined by the concentration of specific types of carotenoids deposited in tissue. In turn, the color produced by a specific carotenoid (usually ranging from yellow to red) is determined by its chemical configuration. Apart from this, the chemical structure of carotenoids results in their poor solubility in aqueous solutions. Importantly, this effectively restricts them to hydrophobic regions of biological systems. But, more importantly, the chemical structure of carotenoids, particularly, the number of double bonds and the type and position of substituent groups, is also responsible for physiological functions, including their antioxidant properties.[11,12]

This capacity of carotenoids to quench ROS forms the basis of the "antioxidant role" hypothesis. But, what are ROS? The term ROS covers both free radicals (*e.g.*, superoxide, hydroxyl, nitric oxide) and non-radical oxidants (*e.g.*, hydrogen peroxide, hypochlorous acid, singlet oxygen). From a biological perspective, the most relevant point here is that ROS are highly reactive and can damage biologically relevant molecules, such as DNA, proteins and lipids.[13]

Most of endogenously produced ROS (about 90%) are normal by-products of mitochondrial activity during aerobic metabolism.[13] Apart from cell metabolism, another important source of ROS is the immune response. During an infection, the immune system cells are activated, which usually implies a certain degree ROS production.[13–15] For instance, lymphocytes constantly generate ROS as a way to combat invading pathogens, whereas macrophages and neutrophils phagocytose and destroy foreign particles through an oxidative mechanism termed respiratory burst that also involves the production of ROS. Even though ROS are produced as part of the killing mechanism, this may be potentially harmful for the host organism, increasing the cost of immune response.

To fight ROS and protect themselves from oxidative damage, organisms rely on a relatively complex antioxidant system composed of endogenously produced compounds, including low molecular weight antioxidants, enzymes and some other proteins without enzymatic functions, plus some

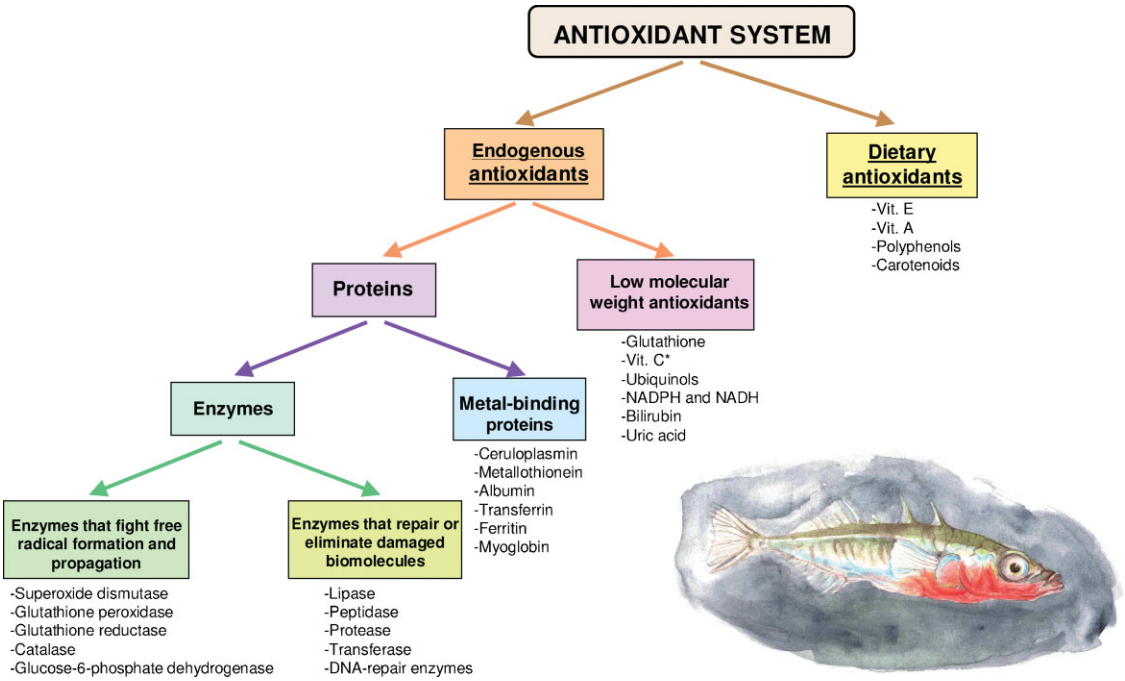

Figure 2. Simplified representation of the main components of the antioxidant system that protects individuals from oxidative damage. (Some constitutive factors that may contribute to antioxidant protection, such as fatty acid membrane composition, have been omited for clarity.) The antioxidant system comprises mainly endogenously produced compounds, such as enzymes that repair or discard molecules that have suffered oxidative damage, plus some endogenous low molecular weight antioxidants. In addition, some other endogenous proteins without enzymatic functions (metal-binding proteins) withdraw transition metal ions from circulation to protect them from reacting with ROS (which may lead to chain reaction and enhanced ROS production). Note that dietary antioxidants, where carotenoids are included, constitute only a small group of all antioxidant compounds that help to fight ROS. This must be kept in mind in those studies aiming to assess the activity or response of antioxidant defenses by measuring just a single component (*Vitamin C may be endogenously produced by some groups, but not by others).

food-derived antioxidants (Fig. 2). The imbalance between ROS and antioxidant defenses is defined as oxidative stress, and is involved in relevant processes such as ageing and several degenerative diseases.[13,16] Within an ecological and evolutionary context, oxidative stress may play a key role in life-history evolution because increased oxidative damage is likely to be a significant constraint in many biological processes.[7,17–19]

Vitamins E, A (which can be derived from some carotenoids by metabolic transformation) and C, polyphenols and carotenoids are the main food-derived antioxidants (Fig. 2). Although dietary antioxidants constitute just another piece within the antioxidant network (Fig. 2), they have been the target for ecologists since their bioavailability is likely to be limited in comparison with other endogenously produced antioxidants, ultimately constraining individual homeostasis. However, there is scant evidence supporting the assumption that dietary antioxidants (such as carotenoids) are limiting in animals and whether this applies to different taxa.[20] Furthermore, we do not really know whether individuals can compensate for a lack of food-derived antioxidants by up-regulating the production of endogenous antioxidant mechanisms, or what the cost of such up-regulation would be.

In vitro studies reveal that carotenoids are able to scavenge ROS. Although carotenoids are not necessarily destroyed after quenching (they can be recycled several times with the participation of other antioxidants; see below), the ''antioxidant role'' hypothesis assumes that carotenoids contribute significantly to ROS scavenging in the organism, and that carotenoids consumed in that function are no longer available for ornament coloration. Hence, an increase in ROS production will lead to reallocation of available carotenoids to antioxidant functions, setting the basis for the hypothetical sequence of increased ROS levels, oxidative stress, decrease in circulating carotenoids and subsequent decrease in carotenoid-based trait coloration. Hence the fact that carotenoid-based traits may reflect the health status (levels of oxidative stress or immune system activation) of the individual.[1,2,6,7]

Re-evaluating *a priori* assumptions: can we extrapolate conclusions from humans to other taxa?

During the last two decades several studies from the medical and nutrition literature summarized and highlighted the antioxidant properties of carotenoids and the beneficial

effects of carotenoid-rich diets on several diseases attributed to oxidative stress.[13,21] However, it should be noted that years before the controversy reached the field of evolutionary ecology, the criticisms regarding the antioxidant properties of carotenoids had also emerged in medical literature.[12,22,23] In spite of this, ecophysiologists and behavioural ecologists have extrapolated the supposed antioxidant role of carotenoids from humans to birds and other taxonomic groups. But, how accurate are these extrapolations? There are radical differences between taxa that makes it risky to extrapolate conclusions from one group to another. The most obvious difference, as highlighted a decade ago by Hill,[24] lies in the fact that mammals and other taxa, such as birds, circulate different amounts of carotenoids. Humans circulate less than 1 μg/mL carotenoids in the blood,[25,26] whereas some bird species may circulate up to 75 μg/mL, with average levels of ~20 μg/mL in many species.[27] One may infer that these higher concentrations would imply greater contribution of circulating carotenoids to antioxidant protection in birds compared to humans. However, it is known that carotenoids may lose their antioxidant effectiveness (or even show pro-oxidant properties) at high concentrations.[12,28] However, although many supplementation studies in birds have elevated circulating carotenoids up to saturation levels (*e.g.*, above 100 μg/mL),[29] except in one case,[30] the effects on health status have been consistently positive, which does not support the hypothesis proposed by some authors that carotenoids may be harmful for the organism and that carotenoid-based signals indicate the detoxification capacity of the individual.[1,2,8]

Humans and other taxa also differ in the specific types of carotenoids that they accumulate and circulate in the blood. Humans tend to accumulate preferentially carotenes (lycopene and β-carotene constitute approximately the 70% of total carotenoids circulated in the blood).[25,26] In contrast, most bird species, for instance, accumulate xanthophylls (specially lutein and zeaxanthin), which may represent up to >90% of the circulating carotenoids.[10] However, only small concentrations of carotenes are found in the avian plasma and body stores, except in those species whose diet is particularly rich in these types of carotenoids.[10] Similarly, species belonging to other taxa (fishes, insects, reptiles) may also strongly differ in their specific carotenoid affinity. This is a relevant issue because, at least *in vitro*, carotenes show a higher ROS scavenging capacity than xanthophylls.[11,31] In addition, the antioxidant capacity exhibited by a particular carotenoid is strongly dependent of the relative abundance of other types of carotenoids present in the sample.[31]

Another factor to consider is that there are physiological differences between taxa that may affect the relative importance of each component of the antioxidant system. For instance, birds, in contrast to mammals, are uricotelic and therefore maintain blood levels of uric acid three times higher than humans. Uric acid may have deep effect on the "antioxidant environment" of the organism,[18] explaining between 36% and 65% of the variation in plasma antioxidant capacity of birds.[32–34] This is also important because the antioxidant capacity of a given molecule is strongly affected by the physiological context in which it is immersed, especially by the concentration of other antioxidants.[35] For instance, vitamin C recycles the tocopheroxyl radical generated by vitamin E after ROS scavenging, and a similar cooperative relationship has been established between vitamin E and β-carotene, and between β-carotene and vitamin C.[36] As a result, many antioxidants have a synergistic (rather than simply additive) effects and it is possible that this kind of relationship applies to many other antioxidants, carotenoids included. Hence, as the antioxidant context varies accross taxa, it seems risky to extrapolate the relative contribution of carotenoids (or any other dietary antioxidant) across different taxonomic groups.

Finally, susceptibility to oxidative stress may differ between groups or species due to differences in metabolic rates or life histories, or due to structural factors, such as the degree of unsaturation of lipids in cell membranes,[18] resulting in different requirements of specific antioxidants. Focusing on carotenoids, it is quite relevant that mammals, for instance, do not possess ornaments to be pigmented by carotenoids and do not need to allocate disproportionate amounts of these pigments to egg yolk formation. This implies different demands and constraints in mammals as compared with other taxa.

Assessing the physiological roles of carotenoids in animals

We can therefore conclude that the initially highlighted antioxidant role of carotenoids can not be extrapolated from humans to birds, fishes, reptiles or invertebrates. Therefore, the *a priori* assumption that carotenoids play a relevant role in the antioxidant system of birds and other taxa needs to be ascertained. In birds, recent studies have started shedding light on this topic. Costantini and Møller[9] reviewed the published literature relating carotenoid levels and any variable indicative of oxidative stress (*i.e.*, antioxidant capacity or oxidative damage), concluding that carotenoids are minor antioxidants for birds. Unfortunately, the scant literature focused on this topic (14 studies), together with a narrow range of species studied ($n = 6$), limited their data set. It is known that the antioxidant system of birds shows important interspecific variations[37], partly explained by differences in life-history traits.[17] Also, the amount of carotenoids (as well as the relative concentrations of each carotenoid type) between species is highly variable, being strongly conditioned by factors such as phylogeny, diet, body size or presence and extent of carotenoid-based ornamentation.[10,27] Although such a limited data set is hardly representative of the whole

Aves class, a recent study reached similar conclusions after analyzing samples from a larger number of species,[37] finding weak and inconsistent relationships between carotenoids and antioxidant capacity. However, these results must be interpreted with caution as infering oxidative stress levels from measures of antioxidant capacity alone may be misleading.[38] Also, we cannot exclude the possibility that the effect of lipophilic compounds like carotenoids is more evident in tissues other than blood (see below). This implies a need to perform additional studies, covering a broader range of species and with an adequate methodology to fully assess the antioxidant role of carotenoids in birds and other groups.

Below, I discuss some relevant issues that must be taken into account to design and perform future studies, paying attention to aspects that must be considered when designing experimental protocols, analyzing samples or interpreting results to assess the antioxidant role of carotenoids in a given model species.

Quantifying oxidative defenses and oxidative damage

Oxidative stress is a multifaceted situation and there are several different analytical approaches to capture it. It is possible to measure all four components of oxidative stress (ROS production, antioxidant defenses, oxidative damage and repair mechanisms). Recent studies have reviewed the available methods to assess these components,[19] so that this topic is not dealt with further here. I focus instead on some useful tools to be considered in evaluating the antioxidant role of carotenoids. These tools can be divided into three main groups: measures of particular antioxidants, measures of antioxidant capacity, and measures of oxidative damage.

The quantification of individual components of the antioxidant system, either enzymatic (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase) or non-enzymatic (vitamins A, C and E, uric acid, glutathione, carotenoids) could give some relevant information when used as biomarkers of antioxidant status. However, although they may indicate up-regulation of the antioxidant machinery or overall levels of antioxidant reserves,[19] these measures alone do not tell us much about the oxidative stress level of the individual.[38] Therefore, measures of the antioxidant capacity (*i.e.*, the ability of the sample to quench ROS) are a much more integrative and functional tool to estimate antioxidant potential of a given sample.[39] There are several different techniques for measuring antioxidant capacity, such as oxygen radical absorbance capacity (ORAC), trolox equivalent antioxidant capacity (TEAC) (also called total antioxidan status, TAS) or ferric reducing ability of plasma (FRAP), among others. However, it should be noted that results are not always consistent across assays because each technique measures a slightly different phenomenon.[32,39]

The third group of analytical tools comprises the measures of oxidative damage in some biomolecules. This is usually done by measuring peroxidation products of lipids, proteins or DNA. Lipids are one of the major targets of oxidative stress, and lipid peroxidation can be quantified by means of biomarkers such as isoprostanes and malondialdehyde (MDA). Carotenoids are lipid-soluble molecules, and their antioxidant role is more likely to be relevant in cellular membranes and lipophilic environments.[40] Therefore, oxidative damage in lipids is probably the most useful biomarker to assess their antioxidant properties.

Probably the best analytical approach is to use measures of antioxidant capacity and oxidative damage simultaneously. This could inform us about the relative contribution of carotenoids to antioxidant capacity, and how important carotenoids are in preventing oxidative damage. Furthermore, measures of oxidative damage are recommended because infering oxidative stress level by analyzing antioxidant capacity alone can be misleading (see below).[38] In addition, given the slightly inconsistent results found between methodologies, the use of more than one single type of assay is desirable. These measures could be complimented with the analysis of one or more key antioxidants. For instance, measuring uric acid seems particularly necessary in birds because, as mentioned above, this metabolite strongly affects measures of antioxidant capacity. Measuring some other specific antioxidant compounds or enzymes could be interesting to eliminate a collateral up-regulation (or down-regulation) of other arms of the antioxidant system after manipulating carotenoid levels. For instance, carotenoid supplementation has been shown to increase vitamin E in birds[41,42] or the activities of some antioxidant enzymes in rats.[43] Also, given the synergistic relationships mentioned above, the scarcity of any other limitant antioxidant (*i.e.*, vitamin E or C) could prevent carotenoids from exerting their antioxidant role. These possibilities cannot be discarded unless other specific antioxidants are monitored. Fortunately, most of these techniques require very small sample volumes, which makes it possible to measure several parameters even when the amount of sample that one can reasonably collect is limited.

Going beyond the blood

The above-mentioned assays are usually applied only to blood samples. Blood seems to be a suitable sample for assessing the effect of circulating carotenoids as the blood stream is the transport vehicle for carotenoids and other antioxidants. However, it is not clear to what extent results from blood are representative of other organs and tissues. More importantly, when testing the antioxidant role of carotenoids, it might be sufficient to find an effect in any single tissue, whether it is mirrored by blood parameters or not. This is because strong cell damage in one tissue

(irreversible or not) could potentially cause detrimental effects to the whole organism, with the consequent general loss of functionality and decreased fitness.

Interestingly, there are subtle variations in carotenoid concentration between organs that may reflect a functional strategic allocation.[10,36] For instance, adipose tissue, liver, ovary and retina tend to accumulate high amounts of carotenoids in birds and other animals,[36] and are promising target tissues that deserve further research. In fact, the retina is a tissue in which the photoprotective and antioxidant role of xanthophylls have been highlighted above that of carotenes,[35] leading some authors to highlight the role of retinal carotenoids in birds.[44] Carotenoids are also present in the skin, where they contribute to protection of this tissue from light-induced damage in a process that, again, involves their role as antioxidants.[45]

Sperm is particularly prone to oxidative-induced damage, which could have major implications in sexual selection.[46,47] Carotenoids (and other dietary antioxidants) have been shown to increase sperm quality in fishes and mammals.[20,36] Although there is less evidence of this effect in birds, reported relationships between carotenoid-based ornamentation correlate with sperm quality[48] and the presence of carotenoids in bird ejaculates[49] are encouraging for the study of the antioxidant role of carotenoids in testes and semen.

Apart from the actual function in the body itself, carotenoids have may play a relevant antioxidant role in the egg of many oviparous taxa.[36,50] In fact, most of current empirical evidences of antioxidant protection by carotenoids in birds come from studies on egg yolks and embryos.[36,51,52] This is likely because the egg yolk is very rich in lipids and carotenoids, and the embryo development is accompained by high metabolism and consequently high ROS production. This results in an ideal context for the antioxidant action of carotenoids. Also, the conditions of low oxygen tensions prevailing during embryonic development in the egg seems to enhance the antioxidant properties of carotenoids.[36] Apart from birds, carotenoids are also present in high concentrations in the eggs of many fishes and reptiles[36] and probably also in invertebrates. So their biological action in these tissues should also be tested.

Finally, the finding that mounting an immune response increased carotenoid allocation to immune organs[53] should not be overlooked. Given the association between mounting an immune response and ROS production, future studies must pay attention to the antioxidant role of carotenoids in immune-related tissues.

Measures in challenged *versus* unchallenged individuals

With the exception of a recent study that manipulates oxidative stress itself[54] and setting aside some studies that manipulate oxidative stress by exposing individuals to an immune challenge (discused below), most of our current knowledge about the contribution of carotenoids to antioxidant defenses is mainly based on correlational studies or, at best, on experiments that analyze the effect of carotenoid supplementation on plasma antioxidant capacity or oxidative damage.[9,37] Although these studies are necessary, they may be insufficient to assess the antioxidant role of carotenoids. The antioxidant system is not fully active in unchallenged individuals, but the real capacity of antioxidant defenses is only displayed when facing an oxidative challenge, which applies both to enzymatic and/or non-enzymatic antioxidants.[19,55–57] This may also potentially be the case of carotenoids. It has been shown that birds may mobilize carotenoids from body stores to blood[58] or to immune organs[53] when facing an immune challenge, which could be related to their use as antioxidants. Therefore, two-way experimental designs in which the effect of improved carotenoid levels on the resistance to a standard oxidative challenge is analyzed[29,33,54,59] are most valuable. In addition, the comparison of several types of oxidative challenges (*e.g.*, moderate and constant levels of ROS *vs.* occasional and intensive increases of ROS production) would help to assess the role of carotenoids and other antioxidants under different scenarios.

Taking a dynamic approach

Our understanding of the role of carotenoids would also be enhanced if we take into account the dynamic nature of antioxidant responses. Protocols where only pre- and post-experiment measures are recorded are informative, but we may lose relevant information for understanding the whole underlying process. For instance, as noted above, an oxidative challenge enhances the production of key enzymes and the release of antioxidants from body stores, increasing their concentration above basal levels to counteract ROS.[19,39,55,57] However, if ROS production persists, antioxidant reserves may be depleted and their levels could drop after a certain time period.[57,60] As a result, depending of the timing of sampling, we may find an increase or a decrease in antioxidant levels (including carotenoids) after an oxidative challenge. In addition, this implies that high antioxidant levels may not necessarily be a desirable condition in unchallenged individuals (*i.e.*, a characteristic of healthiness) as it may simply reflect current up-regulation as a result of a situation of oxidative stress.[38] In addition, individuals may differ in the timing, latency or intensity of response and particular antioxidants may differ in their kinetics and depletion times.[32,61] All these relevant aspects could be overlooked unless a dynamic approach is considered.

Keeping this in mind, the *a priori* prediction of the negative relationship between measures of antioxidant defenses and

the degree of oxidative damage may not always be correct. Measures of antioxidant defenses (*i.e.*, antioxidant capacity or levels of particular antioxidants) reflect current state. In contrast, measures of oxidative damage reflect the current or recent past situation of oxidative stress. Therefore, these two parameters may reflect process slightly decoupled in time, preventing us from finding the expected association. Furthermore, in a unchallenged organism, measures of antioxidant defenses do not necessarily reflect the capacity to prevent future damages, which is more likely to be related to levels during up-regulation (that may correlate or not with baseline levels). Again, studies designed under a dynamic perspective must analyze the relationship between measures of antioxidant capacity and levels of particular antioxidants in individuals before being challenged and during up-regulation, and how these values (or their changes) predict the subsequent oxidative damage.

The importance of experiments in captivity and under natural conditions

Experimental manipulations are much more feasible under controlled captive conditions, where relatively complex research protocols can be performed and many potentially confounding variables (such as diet composition, breeding status, infection by parasites, *etc.*) can be controlled for. However, a free-living individual is likely to experience higher levels of oxidative stress due to locomotion, parasitism, breeding effort, or several environmental stressors[57,62–67] in comparison to a captive conspecific. The antioxidant system of free-living individuals is therefore much more likely to be constrained by different ecological factors, intensifying physiological trade-offs, which may result in a stronger effect to a standard oxidative challenge compared to that of a captive individual. Hence, these studies are essential to evaluate the relative importance of the studied factors (*i.e.*, oxidative stress, carotenoid levels) under a realistic scenario.

Some alternatives for the ''antioxidant role'' hypothesis

Do carotenoids need to be significant antioxidants to convey information about oxidative stress?

The ''antioxidant role'' hypothesis (Table 1, Fig. 1) assumes that carotenoids are linked to oxidative stress because they are significant antioxidants, and therefore individuals face an allocation trade-off between ornamentation or other physiological (antioxidant) functions.[1,2,6,7] However, as suggested by Hartley and Kennedy[8] and recently supported by some empirical studies,[63,68,69] carotenoid-based traits may signal oxidative stress levels even if carotenoids are minor antioxidants. According to this hypothesis (Table 1, Fig. 1), carotenoids might not be very efficient in ROS scavenging *in vivo*, but they may in turn be especially sensitive to the effect of ROS, which alter or destroy their chemical structure and pigmentary properties. Hence, preservation of carotenoid color properties, making their use for ornament pigmentation possible, would indicate lower ROS production or the possession of an efficient antioxidant system. However, carotenoids themselves would not be responsible for the antioxidant efficiency being highlighted by the pigmented trait.

Immune response, oxidative stress, and carotenoids

The immunostimulatory properties of carotenoids have also been hypothesized to play a relevant role in the allocation trade-offs, as diseased individuals must allocate greater amounts of carotenoids to immune function, and these are therefore not available for ornament expression (Fig. 1). However, these immunostimulatory properties of carotenoids are usually attributed to their role as antioxidants because immune response is associated to ROS production and carotenoids may enhance it by ROS scavenging, allowing a more effective response.[70–72] Therefore, both functions are usually lumped together.[2,7] In fact, to date, the most common procedure to study the relationship between carotenoids and oxidative stress has been to promote an immune response.[29,33,34,59]

Nonetheless, it should be noted that, with the information currently available, the link between carotenoids and immunity is sounder than that between these two factors and oxidative stress. In fact, carotenoid supplementation usually enhances immune response,[73–75] whereas it rarely increases antioxidant protection.[9] Conversely, it is well documented that immune responses deplete circulating carotenoids,[29,34,75] which is not always linked to increased oxidative damage.[29,34,59]

Taken together, evidence suggests that the immunostimulatory effect of carotenoids may not be necessarily mediated by their antioxidant role[34] (see Table 1). Carotenoids or their derivatives are involved in the activation of thymocites,[76] the expression of immune-related genes,[77] the up-regulation of proteins involved in cell-to-cell communication,[78] and the increase in membrane fluidity,[72] functions of vital importance when mounting an immune response but not necessarily linked to their antioxidant properties. Considering the antioxidant and the immunostimulatory role of carotenoids as different phenomena (that may be associated or not) could reconcile some of the controversial evidence regarding the physiological roles of carotenoids.

This also stresses the necessity of designing experimental procedures that manipulate oxidative stress independently of promoting an immune response. Experimental manipulation

Table 1. Summary of predicted effects of the four most common or feasible experimental treatments (supplementation with carotenoids, supplementation with non-carotenoid antioxidants, immune challenge or increased ROS production) on carotenoids, antioxidant capacity, oxidative damage and intensity of immune response, according to the main hypotheses relating carotenoids and oxidative stress (H1–H4, see also Fig. 1). It should always be considered that the outcome of an oxidative challenge (i.e., whether it causes oxidative damage or not) will depend of the balance between ROS production and antioxidant defenses. As a generalization, I assumed that increased carotenoid levels imply larger amounts of carotenoids available for ornament expression, although this will ultimately depend of individual allocation priorities. Experimental protocols that include two or more treatments in multi-way experimental designs, especially those combining supplementation and challenge protocols, would be particularly useful for discriminating between these hypotheses (see the text for a detailed explanation). It should be noted, however, that the hypotheses H3 and H4 are not mutually exclusive

		Hypotheses			
Experimental treatment	**Response variables**	**H1: Carotenoids are not antioxidants, immunostimulants or sensitive to bleaching by ROS**	**H2: Carotenoids are antioxidants**	**H3: Carotenoids are not antioxidants, but are sensitive to bleaching by ROS**	**H4: Carotenoids are not antioxidants, but have immunostimulant properties**
Carotenoid supplementation	Carotenoids	↑	↑	↑	↑
	Antioxidant capacity	=	↑[a]	=	=
	Oxidative damage	=	↓[a]	=	=
	Immune response[b]	=	↑[c]	=	↑
Non-carotenoid antioxidant supplementation	Carotenoids	=	↑ or =	↑ or =	=
	Antioxidant capacity	↑[a]	↑[a]	↑[a]	↑[a]
	Oxidative damage	↓[a]	↓[a]	↓[a]	↓[a]
	Immune response[b]	↑[c]	↑[c]	↑[c]	↑[c]
Immune challenge[d]	Carotenoids	=	↓[e]	↓[e]	↓[e]
	Antioxidant capacity[g]	↓, = or ↑	↓, = or ↑	↓, = or ↑	↓, = or ↑
	Oxidative damage[g]	↑ or =	↑ or =	↑ or =	↑ or =
	Immune response[b]	·	·	·	·
Increased ROS production[f]	Carotenoids	=	↓[e]	↓[e]	=
	Antioxidant capacity[g]	↓, = or ↑	↓, = or ↑	↓, = or ↑	↓, = or ↑
	Oxidative damage[g]	↑ or =	↑ or =	↑ or =	↑ or =
	Immune response[b]	↓[c]	↓[c]	↓[c]	↓[c]

[a]Predictions made assuming that supplementation with carotenoids or any other antioxidant has an additive effect on antioxidant capacity and is not accompanied by the down-regulation of any other antioxidant mechanisms as a side effect (see main text for further details).
[b]Estimated as intensity of response against a standard exposition to an antigen.
[c]Predictions made assuming that increased oxidative stress have a negative effect on immune responses.[92–94]
[d]Predictions made assuming an increase in ROS production associated to mounting an immune response (see the text for justification).
[e]Predictions refer to total amount of carotenoids present in the body. This does not exclude the possibility that carotenoid concentration may remain unchanged or even increase in some particular tissues as a result of strategic allocation (see the text for further details).
[f]Increased ROS production by means of any protocol not involving mounting an immune response (see the text for further details).
[g]As a result of the dynamic nature of antioxidant response (see text), the effect of the treatment on the variable response depends on the timing of sampling, the ability of the antioxidant system to cope with the oxidative challenge or the final outcome of the antioxidant response.

of steroid hormones such as corticosterone or testosterone may affect metabolic rates and oxidative stress levels.[79–85] However, these hormones may also impair immune function[86,87] and directly affect carotenoid absorption and transport and antioxidant capacity,[67,88,89] collateral effects that may be undesirable for our purposes. Increasing metabolic rates by increasing physical exercise or, in the case of endotherms, exposing individuals to lower temperatures would increase oxidative stress.[57,60,65,90] However, again, collateral effects derived from increased energy expenditure or changes in hormone (*e.g.*, corticosterone) levels cannot be discarded. A promising alternative to these procedures could be the experimental administration of diluted solutions of paraquat or other similar bipyridine.[54,91] This substance specifically promotes the production of superoxide anion ($O_2^{-}\cdot$), which leads to oxidative damage of lipid membranes, allowing us to directly manipulate the oxidative stress of the individual, avoiding many confounding collateral effects.

Conclusion

The antioxidant role of carotenoids in the living organism has generated great interest among behavioral ecologists as a possible basis for physiological trade-offs ensuring the

honesty of carotenoid-based signals. Recent studies have criticized the core assumption that carotenoids are significant antioxidants *in vivo*.[8,9] This controversy may have arisen because ecologists have extrapolated some initial evidence from medical literature (where the antioxidant role of carotenoids is currently also being questioned)[12,22,23] to other taxa. However, there are substantial differences in the physiology and the antioxidant systems that makes it inadvisable to extrapolate some conclusions across taxa. Although current evidence in birds does not support a strong antioxidant effect of carotenoids,[9,37] available studies do not allow us to reject this hypothesis either. Therefore, the real contribution of carotenoids to the antioxidant system on each studied group needs to be ascertained. Experimental designs addressing this question should take into account several important features of carotenoids and the antioxidant network, avoiding too simplistic interpretations of antioxidant responses. For instance, experimental designs must comprise measures of antioxidant capacity and oxidative damage simultaneously, taking a dynamic approach and covering tissues other than just blood. Guidelines and suggestions given in this article should help in designing, performing and interpreting studies aimed at assessing this crucial question, and help us gain a better understanding of the relative importance of carotenoids within the antioxidant system of individuals from different taxonomic groups. This will finally lead us to a better understanding of the physiological mechanisms underlying the honesty of carotenoid-based signals.

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