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FORUM

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CAN BIRD CAROTENOIDS PLAY AN ANTIOXIDANT ROLE OXIDIZING OTHER SUBSTANCES?

¿PUEDEN LOS CAROTENOIDES AVIARES JUGAR UN PAPEL ANTIOXIDANTE OXIDANDO OTRAS SUSTANCIAS?

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Summary.—*Can bird carotenoids play an antioxidant role oxidizing other substances?*

Carotenoids have moderate ionization energies and very high and positive electron affinities. In other words: carotenoids are more likely to induce oxidation than to prevent it. This result is in stark contrast with the common assumption that carotenoids are good antioxidants, an assumption going back to 1932 that has been subject to scant critical scrutiny. Numerous studies, from medicine to behavioural ecology, show that birds benefit from a diet rich in carotenoids. One of their hypothesized beneficial properties is that carotenoids contribute to fight oxidative stress. Among behavioural ecologists, this hypothesis has led to the proposal that carotenoids are the handicap that stabilizes the evolutionary trajectories of many sexual displays: if carotenoids are required to fight oxidative stress, only high-quality individuals will be able to divert them from metabolic pathways to colourful ornaments. But this explanation is currently under siege: a number of recent studies in birds have found little correlation between carotenoid concentrations in tissues and their total antioxidant ability. Our results suggest that the current paradigm needs a

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thorough revision. It is unlikely that carotenoids prevent the oxidation of molecular machinery, but they can nevertheless act as scavengers of free radicals. In particular, they will readily neutralize free electrons that escape from the mitochondrial electron-transport chains. Testing this possibility will require new experimental approaches: carotenoid concentration should correlate with the ability of tissues to absorb free electrons (i.e. to prevent reduction), but not with its antioxidant potential.

Key words: carotenoids, electron transfer, oxidative stress, sexual selection.

RESUMEN.—*¿Pueden los carotenoides aviares jugar un papel antioxidante oxidando otras sustancias?*
Los carotenoides son sustancias que presentan energías de ionización moderadas, pero afinidades electrónicas muy elevadas y positivas. En otras palabras, los carotenoides parecen estar más capacitados para inducir oxidaciones que para prevenirlas. Este resultado contradice la idea común que identifica a estas sustancias como buenos antioxidantes, un concepto que nace en 1932 y que ha sido objeto de muchas investigaciones. Numerosos estudios han demostrado que las aves se benefician cuando la dieta es rica en carotenoides. Esta ganancia en la salud se le atribuye a su poder para combatir el estrés oxidativo. Dentro de la ecología del comportamiento, esta hipótesis ha llevado a la propuesta de que los carotenoides han sido el *handicap* que estabiliza las trayectorias evolutivas de muchos caracteres sexuales. Si los carotenoides se requieren para combatir el estrés oxidativo, sólo aquéllos individuos que estén en buenas condiciones y que posean alta calidad serán capaces de utilizarlos en ornamentos coloreados. Sin embargo, esta explicación empieza a cuestionarse, ya que estudios recientes en aves han encontrado que la correlación entre la concentración de carotenoides en los tejidos y la capacidad antioxidante no es en realidad tan alta como se pensaba. Los resultados que aquí se presentan sugieren que el paradigma asociado a los carotenoides puede necesitar una revisión. No parece probable que los carotenoides prevengan la oxidación de la maquinaria molecular, pero sin embargo sí pueden atrapar radicales libres. En particular, los carotenoides pueden neutralizar los electrones libres que se forman en las mitocondrias. Comprobar esta posibilidad requiere nuevos protocolos experimentales, ya que la concentración de carotenoides debe correlacionarse con la habilidad de los tejidos para absorber electrones libres (es decir, prevenir la reducción), circunstancia que hasta ahora se ha dejado de lado ya que lo que se ha buscado es demostrar el poder antioxidante de estas sustancias.

Palabras clave: carotenoides, estrés oxidativo, selección sexual, transferencia de electrones.

Among birds and other animals, sexually-selected traits often consist of colourful ornaments produced by depositing pigments in skin, beak and feathers. There is ample evidence that females prefer males with larger and brighter ornaments (Hill, 2002). This female preference suggests that colourful traits are signals of quality, and the handicap principle leads us to expect that colourful displays are costly to produce or maintain (McGraw *et al*., 2005) – although the possibility that cost-free signals are evolutionarily stable cannot be dismissed *a priori* (Szamado, 2003).

There are many ways in which colourful ornaments can be costly. Belt (1874), discussing aposematic colouration, remarked that only distasteful or poisonous species could bear the cost of attracting predators with colourful patterns. More recently, researchers have investigated the possibility that the cost of producing colourful displays resides in the pigments that give colour to ornaments. Although the chemical nature of these pigments varies, many are carotenoids (CAR) (Hill *et al*., 2002; Negro *et al*., 2002). Carotenoids are mainly synthesized by algae, higher plants or microorganisms (Goodwin, 1984). Out of more than 600 carotenoids that have been described (Armstrong, 1997), less than 30 have been found in the diet and tissues of nearly 100 species of birds studied to date (Brambilla *et al*., 1999; Hill and McGraw, 2006).

It has been suggested that the usefulness of carotenoids as true signals of male quality in birds stems from the fact that (i) birds are unable to synthesize CAR, (ii) these molecules are in short supply in the diet and (iii) they are required for a number of physiological processes. If CAR represent a scarce resource that birds require for their survival or fertility, only those individuals able to ensure a more than adequate supply of CAR, or those that for some reason are able to do without them, will have the possibility of diverting CAR from their metabolic requirements, using them as signals.

CAR have traditionally been described as antioxidant compounds (Burton and Ingold, 1984; Krinsky, 2001; McGraw, 2006), and it is often assumed that one of their main physiological roles is the eradication of free radicals through their antioxidant properties (Krinsky and Yeum, 2003). In this paper we briefly review the evidence for and against the antioxidant nature of CAR, paying particular attention to recent calculations of their ionization energy and electron affinity (Galano, 2007; Martínez *et al*., 2008). As we will show, these calculations have important methodological implications. In particular, the practice of quantifying correlations between CAR concentration and antioxidant capacity of tissues (Tummeleht *et al*., 2006) is unlikely to tell us much about the physiological role of CAR. The lack of correlation between CAR concentration and antioxidant activity (see Costantini and Møller, 2008 and reference therein) is unsurprising.

There is a vast literature on the putative antioxidant role of CAR (Krinsky, 1989; Palozza and Krinsky, 1992; Edge *et al*., 1997; Martin *et al*., 1999; Krisnky, 2001; Polyakov *et al*., 2001; reviewed in Paiva and Russel, 1999). It seems clear that CAR can play an antioxidant role in vitro (Krinsky 1989; Palozza and Krinsky, 1992; Clarkson and Thompson, 2000; Krinsky, 2001; Krinsky and Yeum, 2003; Mittler *et al*., 2004) although *in vivo*, the evidence is much less conclusive (Krinsky, 1998). Moreover, numerous studies report that increasing CAR consumption mitigates the effects of diseases normally associated with high oxidative stress (Krinsky, 1998). In birds, recent studies have investigated the relationship of antioxidants and physiology (Horak *et al*., 2006; Costantini *et al*., 2006; Alonso-Alvarez *et al*., 2007; Isaksson *et al*., 2007; Cohen and McGraw, 2009) finding contradictory results (see for instance Costantini and Dell'Omo, 2006 and Costantini *et al*., 2007). Several mechanisms have been proposed to explain the antioxidant effect of CAR: (i) quenching singlet oxygen, (ii) electron transfer, (iii) hydrogen abstraction and (iv) addition (Burton and Ingold, 1984; Krinsky, 1989; Edge *et al*., 1997; Krinsky, 2001; Krinsky and Yeum, 2003). The ability of CAR to quench singlet oxygen, in vitro and in vivo, seems undisputed (Krinsky, 1989; Edge *et al*., 1997; Krinsky, 1998; Krinsky, 2001; Young and Lowe, 2001; Krinsky and Yeum, 2003). It should be noted that this mechanism involves physical interactions between CAR and oxygen molecules, in the absence of chemical reactions. Singlet oxygen is a highly reactive excited state of molecular oxygen (O_2). CAR molecules can accept the energy stored in singlet oxygen, which goes back to its ground state (triplet oxygen), thus preventing the reaction of singlet oxygen with other molecules and the beginning of oxidative chain reactions. Thanks to their polyene chain, CAR can easily dissipate as heat the energy absorbed from singlet oxygen. In the other hypothesized mechanisms (electron transfer, hydrogen abstraction and addition), CAR would behave as radical scavengers: they would participate in chemical reactions with free radicals, neutralizing them and preventing their reaction with other molecules. We now concentrate on the possibility that CAR behave as a radical scavenger through electron transfer.

During electron transfer, an electron is transferred from one molecule to another. The molecule which donates the electron becomes oxidized and the molecule which receives the electron becomes reduced. We hypothesize that CAR (and all radical scavengers) could scavenge free radicals either accepting or donating electrons (Martínez *et al*., 2008). But if the idea that CAR might accept electrons is sometimes mentioned as a logical possibility (Britton, 1995), it has seldom been entertained. There is some logic behind this neglect. An oxidant is a substance that tends to oxidize (remove electrons from) other substances. If CAR accept electrons, other molecules must donate them: CAR would be oxidizing other substances. Can CAR play an antioxidant role oxidizing other substances? A priori, it seems more sensible to assume that an antioxidant is a molecule that prevents the oxidation of another molecule by donating one of its own electrons. Good antioxidants are molecules that require little energy to give electrons away. An antireductant is a molecule that prevents the reduction of another molecule by accepting an electron before the other molecule can do so. Good antireductants are therefore molecules that require little or no energy to accept an extra electron. Antireduction and oxidation refer to the same mechanism: acceptance of an extra electron. Our hypothesis is that CAR oxidize free radicals in order to prevent the oxidative stress.

The ability of CAR to react with oxygen was first discussed in the 1930's (Olcovich and Mattill, 1931; Monaghan and Schmidt, 1932), and subsequently studied by other authors (Gaziano *et al*., 1995; Edge *et al*., 1997; Martin *et al*., 1999; Palozza *et al*., 2008). While this literature concludes that CAR are pro-oxidant because they can react with oxygen, we want to stress that, in this context, the term oxidation does not refer to electron transfer processes. Platt (1952) first suggested that electron transfer was one of the main functions of CAR, and electron-donor and electron-acceptor properties of CAR were experimentally confirmed by Mairanovsky *et al*. (1975). Nevertheless, the possibility that the electron-acceptor (antireductant) capability of CAR plays a role preventing oxidative stress has been so far ignored.

A paper by Burton and Ingold (1984) is to some extent responsible for the assumption that CAR are good antioxidants and not good antireductants, and yet, the authors state that "evidence that [β-carotene] actually has antioxidant activity in addition to its ability to quench singlet oxygen is far from compelling" (Burton and Ingold, 1984, p. 570). Burton and Ingold (1984) already reported that, in the absence of singlet oxygen, the antioxidant nature of CAR was only observed at low oxygen partial pressure. Once again, they refer to the reaction of CAR with oxygen, not to the electron donor and electron acceptor mechanism.

The electron transfer process of a molecule can be analyzed using quantum chemistry calculations. Specifically, quantum chemistry can be used to compute the net energy that a molecule requires to donate or accept an electron, and as we have seen these energies are related to the antioxidant and antireductant nature of the molecule, respectively. (Negative energies imply that energy is liberated in the process.) The "vertical ionization energy" is an index of the energy required to remove an electron from the neutral molecule. Galano (2007) calculated the vertical ionization energy of six CAR, and demonstrated that β-carotene, zeaxanthin and lutein are better antioxidants than echinenone, canthaxanthin and asthaxanthin. Thus, it is inappropriate to study the antioxidant potential of carotenoids as if they were all chemically equivalent. Indeed, not all CAR show the same biological capacities (Woodall *et al*., 1996).

We have used quantum chemistry to calculate the antioxidant and antireductant capacity of several CAR (including those studied by Galano, 2007), melatonin, and vitamins A, C and E (Martínez *et al*., 2008). We computed vertical ionization energy as an index of antioxidant capacity and vertical electron affinity, the energy that the molecule requires to accept an extra electron, as an indicator of electron attraction force and antireductant ability. With these values and using recent definitions of electrodonating and electroaccepting powers (Gázquez *et al*., 2007) we can compare CAR with Fluor (F) and Sodium (Na), (F represents a good electron acceptor –good antireductant– and Na a good electron donor –good antioxidant). These values allow us to create a Donor-Acceptor Map (DAM). This tool is useful for classifying any substance in terms of its capacity to donate and accept electrons (Martínez *et al*., 2008). We found that vitamin E is a much better antioxidant than any of the CAR. Although the CAR show considerable variation on their antioxidant and antireductant capacity, they all have stronger electron affinity (higher antireductant capacity) than any of the vitamins tested, and a lower tendency to donate electrons (although the difference between vitamins A and C and some of the CAR in this respect is not so pronounced). Within the CAR, there is a negative correlation between antioxidant and antireductant capacity, and β-carotene has the weakest and astaxanthin the strongest antireductant ability (Martínez *et al*., 2008). The relationship between the tendency to donate and receive electrons among the CAR species is remarkably linear. Interestingly, the CAR with lower antireductant capacity (i.e. β-carotene) are more efficient fighting oxidative stress than the CAR with higher antireductant capacity (Canthaxanthin) (Woodall *et al*., 1996).

Why should ornithologists be interested in the different mechanisms through which CAR can prevent free-radical damage?

If nothing else, for methodological reasons. Early work concentrated in searching for correlations between sexual displays and CAR concentration or looking for effects of CAR supplementation on sexual displays, male attractiveness and several condition indexes (reviewed in Hill and McGraw, 2006). More recently, researchers have addressed the question whether the correlations and effects obtained had anything to do with the antioxidant nature of CAR (Alonso-Alvarez *et al*., 2004; Isaksson *et al*., 2007), and there is a growing tendency to conclude that the antioxidant properties of CAR have little to do with their use in colourful sexual displays (Hartley and Kennedy, 2004; Costantini and Møller, 2008). But we cannot determine whether CAR alleviate oxidative stress if we ignore one of the possible mechanisms through which they operate.

In order to determine whether the signaling role of CAR is related to their antioxidant properties in birds, ecologists have sought for a correlation between antioxidant activity of tissues and their CAR concentration (Blount *et al*., 2002; Horak *et al*., 2006; Cohen *et al*., 2007 among others). Antioxidant activity is quantified adding a known amount of certain oxidant substances and measuring the rate at which these substances are bleached (Cohen *et al*., 2007). Regardless of whether the methods employed are well chosen to study lipid-soluble CAR, these techniques quantify the ability of tissue to donate electrons, and ignore the fact that free radical damage can be prevented through other means. There are at least two reasons why the ability of CAR to donate electrons is a poor index of their antioxidant ability: (i) the only undisputed mechanism through which CAR are known to prevent oxidative stress in vivo, quenching singlet oxygen, does not involve electron transfer and, at least in vitro, (ii) the ability of CAR to scavenge free radicals (through electron transfer or otherwise) is maximum at intermediate CAR concentrations (Young and Lowe, 2001). Therefore, the observation that the electron-donating ability of a medium is uncorrelated with its CAR concentration (Costantini and Møller, 2008 and references therein) does not allow us to conclude that CAR are not used to prevent oxidative stress.

As a final remark and take-home message, we should point out that molecules are not antioxidant or antireductant in absolute terms: the ability of a molecule to oxidize or reduce another substance depends on the electron affinity and ionization energy of both substances. As a rough guideline, molecule X will oxidize molecule Y if the sum of the electron affinity of X and the ionization energy of Y is a negative quantity. The statement that CAR are antioxidant (from the point of view of electron transfer) is meaningless unless we specify the medium to which we refer. What we can do is to compare one chemical species with another. In this respect, there are two important considerations. First, CAR are very diverse molecules, with different electron affinities and ionization energies (Young and Lowe, 2001; Galano, 2007; Martínez *et al*., 2008). It is important to report with which CAR we are working if we are to compare the results from different studies. Second, even if CAR have been shown to posses antioxidant activity in vitro, it seems clear that, as a group, their electron-donating ability is modest, and lower than that of vitamin E (Martínez *et al*., 2008). But CAR are exceptionally good at accepting electrons (Martínez *et al*., 2008): they are electron Hoovers. To us, it seems unlikely that such a remarkable property has not been put to use. We would like to suggest that CAR use their oxidant nature to prevent oxidative stress. This paradoxical result could be accomplished if, for instance, CAR prevented the formation of superoxide ($O_2^{-\cdot}$) absorbing free electrons lost from the electron-transport chains in mitochondria. The formation of superoxide is the first step in a chain leading to the formation of reactive oxygen species (Mittler *et al*., 2004). By accepting free electrons, CAR could therefore prevent the formation of reactive oxygen species, themselves responsible for the initiation of many oxidative chain reactions.

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