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REVIEW

Carotenoid maintenance handicap and the physiology of carotenoid-based signalisation of health

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Abstract Despite a reasonable scientific interest in sexual selection, the general principles of health signalisation via ornamental traits remain still unresolved in many aspects. This is also true for the mechanism preserving honesty of carotenoid-based signals. Although it is widely accepted that this type of ornamentation reflects an allocation trade-off between the physiological utilisation of carotenoids (mainly in antioxidative processes) and their deposition in ornaments, some recent evidence suggests more complex interactions. Here, we further develop the models currently proposed to explain the honesty of carotenoid-based signalisation of heath status by adding the handicap principle concept regulated by testosterone. We propose that under certain circumstances carotenoids may be dangerous for the organism because they easily transform into toxic cleavage products. When reserves of other protective antioxidants are insufficient, physiological trade-offs may exist between maintenance of carotenoids for ornament expression and their removal from the body. Furthermore, we suggest that testosterone which enhances ornamentation by increasing carotenoid bioavailability may also promote oxidative stress and hence lower antioxidant reserves. The presence of high levels of carotenoids required for high-quality ornament expression may therefore represent a handicap and only individuals in prime health could afford to produce elaborate colourful ornaments. Although further testing is needed, this 'carotenoid maintenance handicap' hypothesis may offer a new insight into the physiological aspects of the relationship between carotenoid function, immunity and ornamentation.

Keywords Carotenoids · Ornamentation · Oxidative stress · Testosterone · Trade-off

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Current view

Many conspicuous ornaments in animals are pigmented with carotenoids. Especially in birds and fish, where the carotenoid-based ornamentation has been widely studied, substantial variability among individuals often exists in the elaboration of these colourful sexual traits (Andersson 1994). This variability is commonly believed to be related to the health of the individual, and therefore, carotenoid-pigmented colouration is assumed to be an honest indicator of an individual quality (Badyaev and Hill 2000). Despite an extensive theoretical background and a considerable experimental effort over the past two decades, the physiological mechanism linking carotenoid deposition with general condition and anti-parasite resistance remains unresolved. Several hypotheses have been proposed to explain the honesty of the carotenoid-based ornaments: (1) Since animals are unable to synthesise carotenoids (McGraw 2006a), the ornamentation may reflect an individual's health as a function of its ability to access sufficient sources of these pigments (Hill 1992; Lozano 1994); (2) the energy demands necessary for carotenoid metabolisation into pigments suitable for deposition into ornamental structures could ensure that only good-health

individuals can afford the investments (Hill 2000); and finally and most importantly, (3) carotenoids are beneficial to the individual, probably in particular due to their antioxidative potential (Alonso-Alvarez et al. 2004; Peters 2007; McGraw and Ardia 2007). According to this view, carotenoids are irreversibly destroyed in the antioxidative processes (Vershinin 1999; Kiokias and Gordon 2004), and hence, there should be a trade-off between their physiological utilisation and their deposition into ornamentation (Møller et al. 2000; Hõrak and Saks 2003).

Recently, a comprehensive model has been proposed suggesting that the trade-off between immunity and sexual signalling is mediated by the interactions of testosterone with carotenoids (Peters 2007). This model is based on the oxidation handicap hypothesis, which proposes that testosterone mediates the trade-off between the ornament expression and free-radical disposal (Alonso-Alvarez et al. 2007; Alonso-Alvarez et al. 2008). While testosterone promotes oxidative stress (possibly through its effect on metabolic rates; Wikelski et al. 1999; Buchanan et al. 2001; Alonso-Alvarez et al. 2007), the expression of many carotenoid-based sexual traits is known to be testosterone dependent, and elevated testosterone concentrations may increase the bioavailability of carotenoids for the ornamental structures (Blas et al. 2006; McGraw et al. 2006; McGraw 2006b but see Alonso-Alvarez et al. 2008). This model pointed out that the additional carotenoids might in turn compensate for the immunosuppressive effect of testosterone. According to this view, the immune system competes with ornaments for the available pool of immunostimulating carotenoids; therefore, ornament elaboration is expected to be determined by the balance between the positive effect of testosterone on the bioavailability of carotenoids and the negative effect of testosterone on the oxidative state and immunity. However, some recent findings have questioned the antioxidative function of carotenoids (Isaksson et al. 2007; Isaksson and Andersson 2008; Costantini and Møller 2008) and even their harmful effect on the health state was proposed (Hartley and Kennedy 2004; Zahavi 2007). Therefore, in this paper, we further develop this alternative concept and argue that under certain conditions honest signalling of individual quality via carotenoid-based ornaments may be preserved by a mechanism that does not rely on the antioxidative function of carotenoids.

Hazardous carotenoids?

Carotenoids group more than 600 different substances with similar biochemical properties (Britton 1995). These pigments are widespread in nature as well as in animal food items and are accumulated in animal (e.g. bird) bodies in large quantities (Hill 1999; Møller et al. 2000). Carotenoids are well known to be immunostimulatory (Chew 1993; Hughes 2001; McGraw and Ardia 2005, but see e.g. McGraw and Klasing 2006) and otherwise physiologically essential (Olson and Owens 1998; Palozza et al. 2006). Although there is substantial evidence documenting their antioxidative potential, the mechanisms of the antioxidative processes are better understood in *in vitro* systems than *in vivo* (see, e.g. Foote and Denny 1968; Burton 1989; Bendich 1989). Hence, conditions of their protective function in living animals are open to discussion (Britton 1995; Hughes 2001; Hartley and Kennedy 2004; Tummeleht et al. 2006; Palozza et al. 2006; Hõrak et al. 2007; Isaksson et al. 2007; Pérez-Rodríguez et al. 2008). Importantly, it has not been proved that carotenoids alone play the central role in the protection against reactive oxygen species (Hartley and Kennedy 2004; Pérez-Rodríguez et al. 2008; Costantini and Møller 2008; Monaghan et al. 2009; Cohen and McGraw 2009). Although definitely valuable, there are many more antioxidative substances forming the antioxidant system of an organism (Catoni et al. 2008; Monaghan et al. 2009).

Considering the known synergistic actions among antioxidants (Liu et al. 2004; Jeon et al. 2008; Monaghan et al. 2009; see also Cohen and McGraw 2009), the antioxidative potential of carotenoids may be only preserved in combination with other dietary-obtained antioxidative molecules such as vitamin C and E (Sies 1993; Brigelius-Flohe and Traber 1999; Liu et al. 2004; Traber and Atkinson 2007) or with endogenously produced antioxidative components (Murphy and Sies 1991; von Schantz et al. 1999). A similar mechanism is known for vitamin E in which antioxidative activity is dependent on the presence of carotenoids (Monaghan et al. 2009). This view is supported by some recent findings providing evidence that carotenoids, including lutein and zeaxanthin used as a base for ornament expression in many animal taxa (McGraw et al. 2003; Andersson et al. 2007), may (at least under some physiological circumstances) increase rather than decrease the negative impact of reactive oxygen species production and thus induce or enhance oxidative stress (Siems et al. 2005; Rao and Rao 2007; Kalariya et al. 2008; see also Monaghan et al. 2009).

The switch from antioxidative to prooxidative behaviour of carotenoids is probably dependent on oxygen concentrations (Edge and Truscott 1997; Palozza 1998; Salerno et al. 2005). The unstable long aliphatic chains of carotenoids are easily attackable by free radicals (Britton 1995), and the products of this cleavage can be oxidised further (Handelman et al. 1991; Mordi et al. 1993; Siems et al. 2005; Prasain et al. 2005). The resultant highly reactive apocarotenals are directly cytotoxic (Siems et al. 2000; Hurst et al. 2005; Alija et al. 2006; Yeh and Wu 2006). It has been shown by Siems et al. (2002) that severe tissue damage

may arise from the presence of carotenoids in cells during the respiratory burst accompanying the inflammatory immune response. The carotenoid cleavage products are also known to be growth inhibitory, proapoptotic and immunosuppressive (Nara et al. 2001; Nagao 2004; Salerno et al. 2005; Kalariya et al. 2008). Thus, carotenoids maintained in tissues exposed to high oxidative stress are potentially hazardous to the individual, and, consequently, the basic assumption of the carotenoid function in the so far proposed trade-offs enhancing honesty of carotenoid-based ornamentation may not be fulfilled in many cases.

Carotenoid maintenance handicap

Hartley and Kennedy (2004) proposed that carotenoids might indicate the presence of other, non-colourful antioxidants in the organism. They suggested that the lack of these protective substances under oxidative stress causes degradation of coloured carotenoids into colourless products. As highlighted above, these carotenoid derivates may even be harmful to the organism. This possibility was also hinted earlier by Zahavi (2007) who was the first to suggest the prooxidative potential of carotenoids. Here, we broaden the theoretical concept of 'risky' carotenoids (*sensu* Olson and Owens 1998) into a new hypothesis on the role of carotenoids in health status advertising. We propose that honesty of the signalisation via carotenoid-pigmented ornaments may be preserved by the balance between the positive function of carotenoids and the detrimental effect of non-colourful carotenoid cleavage products (left vs. right side in Fig. 1). This balance depends on the availability of antioxidative reserves: when these are low, carotenoids are preferably degraded. Moreover, we hypothesise that testosterone represents a double-edged sword in this relationship: although ornamentation enhancing, it simultaneously increases oxidative stress leading to the reduction of antioxidant reserves and eventually to carotenoid cleavage. Thus, testosterone promotes a potential handicap from the maintenance of carotenoids. This mechanism is assumed to be responsible for the assurance of the honesty of carotenoid-based signalisation. In contrast to previous hypotheses, we propose that individuals should optimise carotenoid intake rather than maximise it. Although maximal intake would be beneficial for ornament elaboration and some physiological functions, the maintenance of individually specific balance (and hence carotenoid concentration) is important because of the risk of intoxication with carotenoid derivates.

As can be clearly seen from Fig. 1, we are far from claiming that carotenoids are not (under some circumstances) beneficial for the organism as a whole and for the immune system in particular (see Olson and Owens 1998). It is even probable that in healthy animals they play a significant role in antioxidative processes, perhaps synergistically with other antioxidants (Edge and Truscott 1997; see Fig. 1, left side). Any negative effects of carotenoids in healthy individuals thus could be only minor since the cleavage is inhibited by the presence of other antioxidative substances such as vitamin C, E (Liu et al. 2004) or enzymes (e.g. catalase, superoxide dismutase or glutathione peroxidase; Palozza 1998). Nevertheless, a very different situation might arise in malnourished individuals with elevated carotenoid levels (due to carotenoid accumulation for subsequent deposition in ornaments or due to experimental supplementation) as well as in individuals in oxidative stress (e.g. during an immune response to disease) when the reserves of antioxidative substances

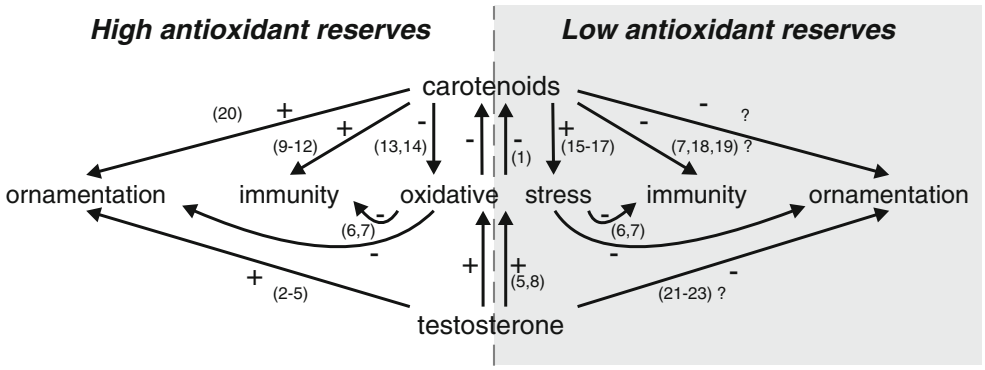

Fig. 1 The hypothetical relationship between testosterone and carotenoids with respect to available reserves of antioxidants. *Plus* and *minus signs* indicate predicted positive and negative associations. *Question marks* are added to pathways requiring further investigation. References: *1* Alonso-Alvarez et al. (2004), *2* McGraw et al. (2006), *3* McGraw (2006b), *4* McGraw and Ardia (2007), *5* Mougeot et al. (2009b), *6* Peters (2007), *7* Salerno et al. (2005), *8* Alonso-Alvarez et al. (2007), *9* Chew (1993), *10* Hughes (2001), *11* McGraw et al. (2005), *12* Clotfelter et al. (2007), *13* Foote and Denny (1968), *14* Pike et al. (2007b), *15* Siems et al. (2005), *16* Mordi et al. (1993), *17* Prasain et al. (2005), *18* Siems et al. (2000), *19* Siems et al. (2002), *20* McGraw (2006a), *21* Stoehr and Hill 2001, *22* Siitari et al. (2007), *23* Alonso-Alvarez et al. (2008)

needed to intercept radicals generated by carotenoid degradation may be depleted. In these cases, carotenoids may not be in balance with antioxidant reserves, and the danger of their harmful effect increases. In other words, we propose that although carotenoid intake may prevent the occurrence of some diseases (Rao and Rao 2007), when a disease or oxidative stress is already present, it may be adaptive for a malnourished individual to maintain only low levels of carotenoids. It follows that only individuals in a prime antioxidative state could afford the presence of high levels of testosterone, which increases the bioavailability of carotenoids, as well as colourful, non-degraded carotenoids in their bodies. Therefore, only high-quality individuals preserve the extensive elaboration of carotenoid-based ornaments. This process ensures honesty of signalisation as any cheaters would suffer from intoxication from carotenoid cleavage products (Fig. 1, right side).

There is also the possibility that carotenoids may be costly for reasons other than their potential toxicity (Olson and Owens 1998). For instance, increased carotenoid input might stimulate some energetically demanding processes, such as carotenoid absorption, transport, storage or transformation, resulting in energy efflux away from some other essential functions. In this case, low-quality individuals would suffer from carotenoid intake without any direct harm caused by toxic carotenoid cleavage products. However, although this scenario may be valid, thus far, it lacks any convincing experimental support. On the contrary, much of the available evidence indicates that many of these processes (e.g. absorption and transportation) are in fact due to the carotenoid lipid solubility completely passive and thus costless (see e.g., reviews by Parker 1996; Furr and Clark 1997; and Spencer et al. 2006).

It could also be argued that the deposition of carotenoids into changeless tissues such as bird feathers would represent a suitable strategy for avoidance of any negative effects caused by these pigments. However, the growth of any tissue (including feathers) is inevitably connected with increased metabolism at the growth site and potential risk of oxidative stress in the tissue (see, e.g., Nagy 2000; Rollo 2002). Allocation of carotenoids to the growing feathers when they are unprotected by other antioxidants could increase the harmful impact of carotenoids on health without any positive effect on the feather colouration. Moreover, as carotenoid cleavage products may inhibit tissue growth (Nara et al. 2001; Nagao 2004), this would further impair ornament expression. It is, furthermore, worth of mentioning that not only feathers but also other carotenoid-coloured ornaments may honestly signal an oxidative state (e.g. ramphotheke, skin and iris; see Faivre et al. 2003; Pérez-Rodríguez and Vinuela 2008) and that these tissues are less stable than feathers.

Supporting evidence

In order to arrive at this ‘carotenoid maintenance handicap’ hypothesis, we assume that: (1) Maximal elaboration of the carotenoid-based sexual ornaments is preferred by the signal recipient. (2) The handicap caused by formation of the carotenoid cleavage products is strong enough to influence the overall physiology of the individual or at least the expression of the ornament. (3) The frequency of carotenoid cleavage is dependent on the body reserves of other protective antioxidants. (4) Testosterone promotes ornamentation but at the same time lowers the protection of carotenoids by other antioxidative molecules. If these presumptions are met, a physiological trade-off between carotenoid maintenance for the use in ornamental traits and their discharge out of the body can be predicted.

Although there has been no direct experimental effort devoted directly to the testing of these predictions, the four main cornerstones of the hypothesis seem to be supported by the evidence currently available.

Preference for maximal elaboration of the carotenoid-based ornaments

There is a huge body of evidence showing that there is a positive selection for individuals with maximally elaborated carotenoid-based ornaments in the process of mate choice in a wide variety of species. As this topic has been recently reviewed in detail elsewhere (see, e.g. Magurran 2005; Hill 2006), we will not include further details here.

Carotenoid cleavage products and animal health

Although virtually nothing is currently known about the influence of carotenoid cleavage products on ornamentation, the existing evidence in other fields seems to be in concordance with our premises. As has been shown in previous sections, there is no doubt about the real fact of carotenoid cleavage and the toxicity of the cleavage products. The same which was documented earlier for carotenoids in general is probably true in particular even for the most abundant carotenoids utilised in ornament expression, i.e. lutein and zeaxanthin (Prasain et al. 2005; Kalariya et al. 2008). Less clear is to what extent this process takes place in living animals. Recent research on carotenoid supplementation in humans and other primates, however, shows that carotenoid supplementation significantly increases the quantity of the carotenoid cleavage products in various tissues (Khachik et al. 1997; Khachik et al. 2006; Ho et al. 2007). This raises a question if such carotenoid cleavage may impair the individual health. Although evidence of this kind is still only limited, a conditionally negative effect of carotenoid supplementation

on health is known. Several human clinical studies (e.g. Heinonen et al. 1994; Omenn et al. 1996) provide evidence that supplementation with particular carotenoids in individuals exposed to oxidative stress (smokers) increase the incidence of lung cancer and risk of death (see also Peters et al. 2007). Recent research on the molecular mechanism involved indicates the key role of carotenoid cleavage products in this process (Yeh and Wu 2006). A different kind of evidence comes from yeast studies. In mutants with impaired endogenous antioxidative system, an erratic and dose-dependent effect of carotenoid supplementation on cell reactive oxygen species levels has been found (Amari et al. 2008). We clearly need more research in this field, especially in non-mammalian vertebrate species. Nevertheless the potential negative impact of carotenoid cleavage on health should not be overlooked.

Non-colourful antioxidants and ornamentation

Consistent with Hartley and Kennedy (2004), the 'carotenoid maintenance handicap' hypothesis predicts a positive influence of non-colourful antioxidants on ornamentation. This relationship was supported by Bertrand et al. (2006) who showed that non-colourful antioxidants added to the diet may have a significant positive impact on carotenoid-based bill colouration in zebra finches when combined with carotenoid supplementation. Similar findings were also described by Pike et al. (2007a) in fish and by Pérez et al. (2008) in gulls. The results of these studies indicate that, in agreement with our predictions, carotenoid maintenance may be dependent on available reserves of non-colourful antioxidants. When these are replete, carotenoid accumulation is no longer beneficial. However, it must be noted that there is also some evidence of a similar kind that seems to contradict the predictions of the 'carotenoid maintenance handicap' hypothesis. For instance, the results of Hõrak et al. (2007) and Karu et al. (2008) do not indicate any positive effect of vitamin E supplementation on ornamentation and antioxidative potential when added to a carotenoid-rich diet. Yet, as Hõrak et al. (2007) admit, it is difficult to judge whether the levels of this vitamin in the experimental individuals were low enough to allow the detection of a positive effect of supplementation. If they were not, vitamin E supplementation could have no or an adverse effect (exhibiting a similar prooxidative effect as in carotenoids; Monaghan et al. 2009). Moreover, vitamin E may act only in combination with other antioxidants (see, e. g. Liu et al. 2004) or antioxidants other than vitamin E (such as those of the endogenous origin) could be important in carotenoid protection (see Cohen and McGraw 2009). Therefore, these findings cannot be easily viewed as a direct disproof of the 'carotenoid maintenance handicap' hypothesis.

Testosterone regulation of carotenoid physiology

The importance of hormonal regulation (probably mediated by testosterone) in the proposed process needs to be supported. Though it cannot be currently fully clarified, here we review the most important results of recent research. As proposed, ornamental colour is associated with body carotenoid levels, often judged from plasma (e.g. Mougeot et al. 2009a). It has been recently found in the red-legged partridge (*Alectoris rufa*) and the zebra finch (*Taeniopygia guttata*) that testosterone increases the levels of these carotenoids that may serve for the ornament elaboration (Blas et al. 2006; McGraw et al. 2006). Higher levels of plasma carotenoids have also been reported after testosterone treatment in red-legged partridge males by Alonso-Alvarez et al. (2008). However, although difficult to measure, evidence from the zebra finch and red-legged partridge suggests that at the same time testosterone may intensify the oxidative load and potentially cause oxidative stress (Alonso-Alvarez et al. 2007; Alonso-Alvarez et al. 2008; Mougeot et al. 2009b). These results thus suggest the equivocal effect of testosterone on physiological processes influencing ornamentation. This view is supported also by the known U-shaped relationship between testosterone levels and ornamental colouration in zebra finch females (McGraw 2006b).

Further evidence

If viewed independently on the current paradigm, additional (but less direct) evidence supporting the hypothesis of the 'carotenoid maintenance handicap' can be found. For example, as carotenoid supplementation increases the amount of carotenoid cleavage products *in vivo* (Ho et al. 2007), animals may be expected to try to optimise by some physiological mechanisms the level of carotenoids in their tissues. This could help them minimise the negative effect of the toxic cleavage products. A situation resembling this process was documented by Alonso-Alvarez et al. (2004) who described a carotenoid intake plateau in the zebra finches. As they reported, above certain level, no further carotenoid supplementation led to an increase in plasma carotenoid content or bill colouration. A similar plateau in colouration has also been described in some fish species (Doolan et al. 2009). It is possible that this plateau represents the state in which carotenoids are in equilibrium with other antioxidative substances. Although the exact mechanism of the equilibrium preservation remains unknown, it may be connected with carotenoid intracellular transportation rate in plasma membrane region of duodenal cells (Parker 1996) or with the saturation of carotenoid blood carriers, i.e. lipoproteins (see McGraw et al. 2005; McGraw et al. 2006; McGraw and Parker 2006). Alonso-Alvarez et al. (2004) have also shown that the plateau was

lower under the conditions of stimulated immune function (see also Hughes 2001) and, most importantly, that the carotenoid levels decreased, albeit the carotenoid intake was sufficient. Exactly the same result could be expected if the birds discharged the potentially risky carotenoids under the increased oxidative load due to enhanced immune activity. An analogous decrease of plasma carotenoid levels after a locally induced immune response has also been recorded in red-legged partridge by Pérez-Rodríguez et al. (2008). As the decrease of plasma carotenoids had no effect on the total antioxidant capacity or oxidative stress, Pérez-Rodríguez et al. (2008) suggest only a minor role of carotenoids in the antioxidative protection. These results thus support the hypothesis that carotenoids are expelled from circulation in potentially stressful situations. It has been shown by McGraw and Ardia (2007) that in testosterone unmanipulated individuals carotenoid levels are positively associated with immune function, while the opposite is true in birds with testosterone implants (potentially oxidative-stressed). This decrease in immune function might reflect the negative impact of carotenoid cleavage derivates, indicating the emergence of a potential handicap. The negative effect of carotenoid supplementation on specific immune functions related to oxidative burst was shown earlier by McGraw and Klasing (2006). Thus, though indirect, these results seem to agree with the predictions of the 'carotenoid maintenance handicap' hypothesis but not so clearly with the current paradigm.

The proposed hypothesis also offers an alternative explanation why testosterone treatment did not affect colouration in some studies (Alonso-Alvarez et al. 2007), while in some others, such a treatment even reduced ornamentation in carotenoid-supplemented individuals (Stoehr and Hill 2001; Siitari et al. 2007): Under artificially elevated oxidative load caused by testosterone, handicapping carotenoids are likely to be lowered in ornamental tissues. The clearest example of this kind has been recently given by Alonso-Alvarez et al. (2008). In the red-legged partridge, these authors showed that in testosterone-supplemented males, there is a negative association between ornamental hue, i.e. a positive association between the quantities of ornamental carotenoids and oxidative damage. Although interpreted by the authors in a different, equally well-founded way, these results may also serve as strong support for the view presented herein. Since testosterone promotes both aggressiveness and oxidative stress, our hypothesis could also explain why aggressive males may be less ornamented than the non-aggressive ones (McGraw and Hill 2000).

On the basis of the current evidence, our hypothesis seems to offer a valid explanation for findings of some studies that cannot be interpreted easily using traditional models based on direct antioxidative function of carotenoids. Nevertheless, we are conscious that the contemporary evidence does not entirely cover all interactions expected by the carotenoid maintenance handicap hypothesis (Fig. 1), and further experimental testing is needed to verify its assumptions.

Future prospects

As highlighted in Fig. 1, there are three areas for which more precise evidence is especially needed. In animals with low antioxidant reserves, we need to show that supplementary carotenoids impair the resultant ornamentation (1) and immune function (2). Little is also known about the potential adverse effect of testosterone manipulation on ornament expression (3). Sufficiently strong evidence confirming the existence of these three relationships would disprove the current paradigm and support our hypothesis. To test these relationships, the inclusion of carotenoid-antioxidant imbalance in experimental studies is crucial. Thus, special model designs have to be adopted. We predict that carotenoids are risky especially:

1. in animals under low nutritional condition (as they may also have generally low antioxidant reserves);
2. in individuals fighting a disease (since many immune mechanisms are commonly accompanied by a respiratory burst in phagocytes, increasing the demand for antioxidant reserves);
3. in individuals engaged in elevated levels of physical activity (e.g. breeding individuals or individuals participating in aggressive interactions) as these are associated with increased metabolism and respiration;
4. in growing or moulting individuals (since growing tissue also exhibits an elevated metabolism);
5. in physiologically stressed animals (since stress generally increases metabolic rates).

Therefore, we propose that experimental work should include animals with a long-term stimulated immune function or those under poor nutritional conditions, social stress or elevated metabolic activity. Under the 'carotenoid maintenance handicap' hypothesis, a decrease in carotenoid intake and no effect (or even an adverse effect) of carotenoid supplementation on the elaboration of carotenoid-dependent ornaments and immune function is expected in individuals under any or several of these circumstances. In other words, we predict that among animals with poor antioxidative reserves, carotenoid-supplemented individuals will perform generally worse than control individuals without any carotenoid supplementation.

To increase antioxidant reserves, the supplementation of the treatment groups in experiments should differ in more aspects than simply the presence or absence of supplementary carotenoids. We suggest that some basic antioxidative molecules (such as vitamins C and E, preferably in

combination, see Liu et al. 2004) or even supplementary proteins (substantial antioxidative potential is based on the endogenous production of free-radical scavenging enzymes; von Schantz et al. 1999) should be included. These elements would, in the case of correctness of the hypothesis proposed herein, promote carotenoid-based ornamentation (see prediction 3 of the hypothesis).

The role of testosterone in ornament expression under the proposed conditions should also be clarified. The evidence on carotenoid bioavailability increase after testosterone supplementation needs to be expanded and the precise mechanism further described. This seems to be crucial for the proposed hypothesis. Especially important is the destiny of the carotenoids that appear in blood plasma after the testosterone treatment (Blas et al. 2006). Whether these carotenoids are under the specific circumstances proposed herein deposited into ornamentation or removed from the ornamental tissue and possibly even from body is a key question (for evidence indicating the possibility of the latter scenario see Alonso-Alvarez et al. 2008). Worth of examining is also the effect of testosterone manipulation on levels of carotenoid cleavage products in plasma and (as, e.g., apo-carotenal levels in tissues and body fluids may differ, see Parker 1996) also in other tissues, including ornaments.

As we have already emphasised, carotenoid cleavage products possess their cytotoxic properties at least partly due to their prooxidative potential. Therefore, research focused on testing the predictions of the proposed hypothesis would benefit from involvement of oxidative state measurements and measurements of antioxidative capacity and oxidative damage in various tissues. This may be particularly important for clear differentiation between the current paradigm and the alternative 'carotenoid maintenance handicap' hypothesis. In tissues with other antioxidative reserves depleted (i.e. those with low antioxidative capacity), increased oxidative damage caused by release of reactive oxygen species can be predicted after carotenoid amount increases (e.g. after dietary supplementation) only on the basis of 'carotenoid maintenance handicap' hypothesis and not on the basis of current paradigm. Furthermore, examination of the oxidative state in tissues other than plasma (e.g. growing ornamental tissues, liver or fat) together with analysis of the carotenoid content in these tissues would be useful for determining the direction of carotenoid transport (utilisation versus expulsion). The quantities of carotenoid degradation and cleavage products should also be assessed. However, researchers should consider in their interpretations that it may be problematic to judge the antioxidative capacity (Cohen and McGraw 2009) and the oxidative state (Monaghan et al. 2009) based on a single test adopted.

The experiments directed to testing the 'carotenoid maintenance handicap' hypothesis could also profit from involvement of the behavioural aspect. We may expect the avoidance of food with high carotenoid content by nutritionally restricted individuals compared to controls. It has been shown in some studies that a direct intake of carotenoids, rather than carotenoid reserves in body tissues, contributes to ornament elaboration (Hill 1992). Hence, we predict that low-quality males should avoid carotenoids, especially during energy-demanding and potentially stressful moulting periods (i.e. during the periods of feather ornamentation development). It is also possible, however, that the carotenoid exclusion appears only after the carotenoids enter the body, indicated, for instance, by lowered carotenoid content in plasma, in other tissues or in the growing (and potentially oxidatively stressed) sites. It should be noted that carotenoids circulating in blood represent only a small part of the total volume of carotenoids in the body (Bendich and Olson 1989); consequently, it may be difficult to assess the total body content of carotenoids or their allocation on the basis of widely used blood sampling (Parker 1989; El Sohemy et al. 2002; see also the difference between plasma and ornament carotenoids in Alonso-Alvarez et al. 2008). Examination of the carotenoid content in faeces might be also useful. For instance, in humans, it has been demonstrated that about 45% of lutein intake is not absorbed in the digestive tract (de Moura et al. 2005). If organism possesses the ability to regulate the carotenoid absorption, it might eliminate their potentially negative effect without costs. In that case, we predict more unabsorbed carotenoids in faeces of low-quality individuals (low antioxidative reserves) in comparison with high-quality ones (sufficient antioxidative reserves).

Finally, we expect that the trade-off mechanism inherent in our hypothesis is also testable for physiological activities other than ornament expression. For example, bird eggs are known to contain high levels of carotenoids. These pigments are important to the developing embryo but they are not the only antioxidants deposited into the eggs by mothers (Blount et al. 2000). We hypothesise that females with low levels of other antioxidants (e.g. vitamin C or E) but with a surplus of carotenoids will lay eggs with lower carotenoid content than females with unrestricted reserves of both these components. Moreover, hatching success and/or embryo development rate should be lower in eggs of poorly nourished females with experimentally elevated carotenoid content than in control (non-supplemented) females. Similar trade-offs may be expected whenever carotenoid levels are a sign of oxidative state.

Conclusion

The proposed 'carotenoid maintenance handicap' hypothesis may represent a valuable alternative to the traditional

view of the mechanisms enforcing the honesty of signalisation through carotenoid-based secondary sexual traits. We believe that ignoring the potential trade-offs predicted by this hypothesis in future models could lead to misinterpretation of observed experimental results. Testing our hypothesis could bring some new insights into the rapidly developing theory of signalling function of carotenoid-based ornaments and the evolution of colouration through the process of sexual selection.

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