CAROTENOID-BASED ORNAMENTS AS SIGNALS OF HEALTH STATUS IN BIRDS: EVIDENCES FROM TWO GALLIFORM SPECIES, THE RED-LEGGED PARTRIDGE (*ALECTORIS RUFA*) AND THE RED GROUSE (*LAGOPUS LAGOPUS SCOTICUS*)

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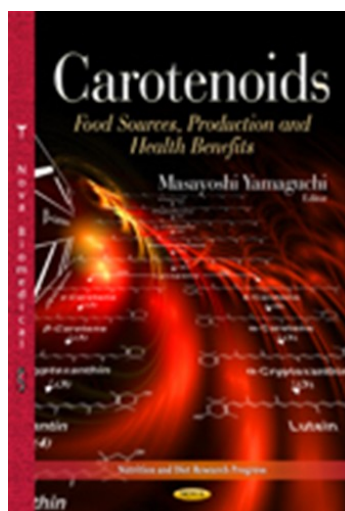
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ABSTRACT

Carotenoids are large lipophilic compounds that can only be produced by plants, fungi and bacteria. Although animals cannot synthesize them de novo, many taxa accumulate them in exposed parts of the body for communication purposes. In many birds, carotenoids are responsible for the bright yellow-red coloration of integuments or plumages, and these have often been shown to advertise an individual's superior health. Some of the hypotheses aimed at explaining the function of carotenoid-based traits rely on the assumption that carotenoids have key physiological roles in the organism. Specifically, they are hypothesized to be significant antioxidants and immunostimulants, being therefore intimately linked to the anti-parasite defenses and health. The mechanisms and functions of the expression of carotenoid-based traits have been particularly well studied in birds. In this chapter we first review the current hypotheses linking carotenoids, oxidative stress, immune response and parasites, together with the current evidences in support of these key physiological functions. Secondly, we focus on two gallinaceous bird species that have been intensively studied in recent years, either in captivity (the red-legged partridge, *Alectoris rufa*) or in the wild (the red grouse, *Lagopus lagopus scoticus*). In both species, ingested carotenoids are metabolized and transformed before being used to color bright red ornaments. The redness of the sexual ornaments relates to the concentration of carotenoids in blood, and varies with infection levels by intestinal parasites and with the ability of individuals to mount an immune response to pathogens. Circulating levels of carotenoids through the blood stream are also negatively affected by physiological stress (oxidative stress and stress hormones), which is partly a consequence of parasite infections and immune responses. The evidence for carotenoids to act as antioxidants and to reduce oxidative stress is still mixed and debated, but some studies highlighted that carotenoid-based ornaments can signal an individual´s ability to resist oxidative stress. Finally, we identify key open questions that deserve further investigation in this field.

Keywords: Carotenoid, red grouse *Lagopus lagopus scoticus*, red-legged partridge *Alectoris rufa*, parasite, immunity, oxidative stress

INTRODUCTION

Carotenoids are a large family of lipophilic compounds produced by photosynthetic organisms and certain bacteria and fungi (Goodwin 1984). Animals are not able to synthesize carotenoids *de novo*, but often deposit these pigments in skin, bills, eyes, scales and feathers (Fox 1976). This results in some of the most striking visual displays (bright yellow-red ornaments) of the animal kingdom. These carotenoid-based colorations are becoming an increasingly popular study subject among evolutionary and behavioral ecologists, as these traits often play key roles in communication (i.e. they are used as signals of bearer quality in social and mate choice contexts). This has been intensively investigated in birds in the recent years, a taxonomical group where carotenoid-based colorations are particularly prevalent and, perhaps, the most common taxon of study for behavioral ecologists. In fact, some avian carotenoid-based traits, such as the red plumage of the house finch (*Carpodacus mexicanus*) or the red beak of the zebra finch (*Taenyopigia guttata*), are amongst the most deeply studied animal sexual signals.

The evolution of carotenoid-based traits into signals of quality requires the existence of a differential expression of such coloration (i.e. size of the color patch or color intensity) for high- and low-quality individuals. Thus, as for any signaling trait, the expression of carotenoid-based colorations must be linked in some way to individual quality in order to

evolve as reliable signals of quality. This drives us to the concepts of "honesty" and "costs". The production or maintenance of honest (i.e. reliable) signals must entail significant costs of different nature (i.e. behavioural, physiological, reproductive, etc.) for the bearer in order to prevent the evolution of individuals producing signals that do not correspond to their actual quality, i.e. cheaters (Zahavi 1975). Thus, honest signals should work as *handicaps* for the organism, so that only high quality individuals are able face the costs asociated with maximal signal expression. Apart from these *revealing handicaps*, honest signaling may also result from the expression of *condition/dependent handicaps* (Collins 1993; Hill 2011), which are traits that do not impose a special cost for the bearer, but whose production is linked to individual condition (health, parasite load, energy reserves) in such a way that only healthier individuals are able to maximize signal expression. As we will see below, most hypotheses addressing the function and evolution of carotenoid-based traits as honest signals of quality refer to condition-dependent handicap mechanisms.

CAROTENOID METABOLISM IN BIRDS: FROM FOOD TO VISIBLE INTEGUMENTS

The above mentioned versions of the handicap principle applied to carotenoid-based traits require a deep knowledge of the physiological pathways followed by carotenoids. It implies the study of the different stages involved from their acquisition via food sources to their deposition in integuments or plumages creating brightly colored signals. Birds must first obtain carotenoids from their diet. However, birds and other animals are able to perform some metabolic transformations, so that the carotenoid composition of visible integuments and internal tissues may differ substantially from that of food sources. Although more than 700 different carotenoids have been described, only a very small subset of them can be found in the organism of birds (McGraw 2006). This is because only some specific carotenoids are present in their diets, but also because absortion efficiency greatly differ depending on the carotenoid type, ranging from 1 to 99% (Parker et al. 1999), as well as the bird species. In general, birds tend to preferentially absorb and accumulate xanthophylls (carotenoids that contain oxygen in their functional groups) over carotenes (which contain only hydrogen). Less than 10 different carotenoids have been described in avian diets (McGraw 2006), but their relative amounts radically differ: about 60-90% of ingested and circulated carotenoids in birds are lutein and zeaxanthin, whereas only small amounts of β-criptoxanthin, β-carotene and others carotenoids are found. Diet composition strongly determines the relative proportion of circulated carotenoids. Aquatic species, for instance, circulate significantly higher amounts of β-carotene, canthaxanthin or astaxanthin than species feeding on seeds or plants (McGraw 2006). As expected, diet explains a significant proportion of the variability in blood carotenoids between species (Tella et al. 2004). Species feeding on plants show higher carotenoid-levels, whereas those feeding on vertevrate preys show lower levels of blood carotenoids. However, beyond such effect of diet, variability in circulating carotenoids in birds (ca. 65%) is mostly explained by phylogenetic relationships: species sharing common ancestors tend to show similar levels of circulating carotenoids (Tella et al. 2004). Although this phylogenetic effect could be due partly to its covariation with diet, it does stress the importance of physiological processes, like absortion or transport efficiency, that are likely conserved and more similar among closely related species.

In vertebrates, ingested carotenoids are extracted from food along with other lipid-soluble molecules by passive diffusion trough the intestine (Parker 1996). In birds, however, there seems to be some lipid-independence in the absorption of carotenoids, and evidence from

chickens indicate that carotenes and xanthophylls are absorbed at different sections of the intestine (Tyczkowski and Hamilton 1986). Once they are retrieved from food and fractioned with lipids in the intestinal mucosa, carotenoids are packaged into chylomicrons, enter the lymphatic system and are circulated through the bloodstream. Lipoproteins are the exclusive transporters of these pigments in the body, and experimental evidence in the zebra finch have shown that increases in lipoprotein levels enhance circulating carotenoids, ultimately increasing carotenoid-based beak coloration (McGraw and Parker 2006). Also, it has been proposed that lipoprotein levels can be upregulated by androgens, setting a basis for the endocrine control of carotenoid-based signals (McGraw et al. 2006). Although the link between circulating carotenoids and androgens has been supported by several different experimental studies in birds (Blas et al. 2006; Alonso-Álvarez et al. 2009; Martínez-Padilla et al. 2010; Peters et al 2012), whether such mediating effect of lipoproteins is a general rule still awaits further studies.

Bounded to lipoproteins, carotenoids are delivered by blood stream to internal tissues, as well as to colored external integuments in species with carotenoid-based ornamentation. Carotenoid concentration among tissues of a given individual are overall correlated, although some tissues and organs (i.e. fat, liver, ovaries in females during reproduction) are more likely to accumulate carotenoids than others (Surai 2002; McGraw and Toomey 2010). The specific carotenoids deposited in body tissues generally match those found in the blood, although some subtle variations between organs have been described, which could reflect some physiological discrimination or tissue specific demands (McGraw 2006).

Carotenoids present in external integuments of birds (skin, beaks, feathers) may come directly from diet, or may have been metabolically transformed prior to deposition. Carotenoid-based traits typically show yellow, orange or red colors depending on the specific types of carotenoids deposited, as well as their relative and absolute concentrations. Yellow colorations result from the deposition hydroxycarotenoids (e.g. lutein, zeaxanthin, canary xanthophylls), which are the carotenoids most commonly acquired through diet. Ketocarotenoids (e.g. astaxanthin, canthaxanthin, adonirubin) are red carotenoids responsible for reddish colorations of plumages and bare parts. These are metabolized from yellow hydroxycarotenoids or from β-carotene. In most cases, a mixture of different carotenoids, with different origins, are found in avian integuments. Also, carotenoids may interact with other pigments (eumelanin, pheomelanin) or with the microstructure of feathers and tissues to produce a large variety of colors, like green or blue (e.g. Lucas and Stettenheim 1972; Prum and Torres 2003). Interestingly, carotenoids deposited into feathers are typically in their free forms, whereas those deposited into living tissues like skin, beaks and combs, are often esterified (Czeczuga 1979; Casagrande et al. 2011; García de-Blas et al. 2011). Carotenoid esterification in these tissues could be related to the dynamic nature of the color of these traits (Pérez-Rodríguez 2008) or could play a role in stabilizing pigment properties. Unfortunately, experimental studies on this topic are still pending.

The specific site where dietary carotenoids are metabolized into colorful integumentary carotenoids have been the subject of debate for some time. The liver was initially proposed as a potential site for carotenoid conversion, given its richness in carotenoids and enzymes (Brush 1990). However, later studies failed to find in blood or liver those metabolically derived carotenoids present in feathers or beaks of songbirds, suggesting that these metabolic transformation might be made directly within the integument or within the follicle of developing feathers (McGraw 2004). Nevertheless, some yellow metabolized carotenoids – e.g. anhydrolutein and dehydrolutein– have been found in the blood of at least one bird species before depositing them into feathers (McGraw and Schuetz 2004). Also, recent studies have found exceptions among red metabolically-derived carotenoids: the red carotenoid 3-hydroxy-echinenone, present in the feathers of common crossbills (*Loxia*

curvirostra), was found in the liver and plasma of the species, but not in its diet, suggesting that the liver was the site of conversion (del Val et al. 2009). Clearly, more studies, covering a larger set of species, are required to elucidate where carotenoids are transformed. Although the role of the liver on integumentary carotenoid metabolim is debated, its role in pro-vitamin A metabolism of ingested carotenoids is well supported, which has been recently vindicated to propose a novel pathway for the honesty of carotenoid-based signals of health (Hill and Johnson 2012). Given the limited knowledge about the site(s) of metabolic conversion, it is not surprising that the identity of the enzyme(s) responsible for these transformations are still unkown to date. The enzymatic control of these conversions remains speculative (Stradi et al. 1996, McGraw et al. 2003, McGraw 2006) and the same applies to our understanding of the underlaying genetic mechanisms (Walsh et al. 2012).

CAROTENOID-BASED ORNAMENTS AS HONEST SIGNALS OF QUALITY

Carotenoids are the second most prevalent pigment in the avian integument, melanins being the first. Unlike these, that may lead either to mimetic or conspicuous patterns, carotenoids mostly lead to striking yellow, orange and red traits. Thus, carotenoid-pigmented plumages, beaks, wattles, combs and other skin patches, which evolved to be seen and play crucial roles in bird communication. Carotenoid-based traits often function as communication signals, but what do they signal? Handicap theory predicts that the information conveyed by a given signal must be linked to the cost or the limitation associated to its production. Evidence from several species support the signaling role of carotenoid-based signals, particularly in sexual selection scenarios (reviewed by Hill 2006). Initial studies proposed that carotenoid-based traits evolved as signals of foraging efficiency and body condition (Slagsvold and Lifjeld 1985; Hill 1990), whose honesty was achieved by a condition-dependent handicap mechanism. This hypothesis relies on the fact that carotenoids must be acquired through diet and the assumption that carotenoids are a limited and scarce resource for most species. Good foragers or dominant individuals are expected to have a better access to food sources, also obtaining higher amounts of carotenoid to maximize signal expression. This implies that carotenoid-based signal expression will be honestly associated with nutritional status, with energy and carotenoid intake being positively associated (although this may not always be the case, e.g. Sternalski et al. 2010). Recent evidence in birds indicate that individuals with brighter carotenoid-based ornaments also show a greater capacity to solve foraging problems (Mateos-González et al. 2011), providing behavioral support for this hypothesis.

This "foraging ability" hypothesis remained for years as the main plausible mechanism to explain the evolution and maintenance of carotenoid-based traits as signals of quality. However, the publication of the seminal paper of Lozano (1994) opened a new window for the study of these traits, highlighting the importance of carotenoids as mediators of several key physiological processes, notably immune responsiveness and parasite resistance. Later, von Schantz et al. (1999) incorporated these ideas within a broader context, highlighting the connections between oxidative stress and immunity and paying attention to the antioxidant properties of carotenoids. This resulted in an alternative hypothesis to explain the evolution and maintenance of carotenoid-based signals, which relies on the assumption that carotenoid pigments have crucial physiological functions for self-maintenance. If so, individuals should potentially face a trade-off between allocating available carotenoids for these self-maintenance functions or for ornamental coloration. In further detail, healthier individuals would require lower amounts of carotenoids for health-related functions, allowing them to allocate more carotenoid pigments to ornamental coloration, to display brighter or more intensely colored signals, and advertise their superior health to conspecifics (Figure 1).

These carotenoid-related physiological trade-offs are usually pooled under the same mechanism to explain honest signalling. This is understandable given that parasites, immune function and oxidative stress are closely interconnected, and that the antioxidant and immunostimulant roles of carotenoids are often difficult to tease appart. However, the implications of these functions differ, and they should be considered separately in order to fully understand the information content of carotenoid-based signals.

CAROTENOID-BASED TRAITS, IMMUNOCOMPETENCE AND PARASITES IN BIRDS

Parasites are a major force in evolutionary biology as they detract energy from their hosts, damaging their health and ultimately their fitness (Møller et al. 1990, 1999). Consequently, hosts should prevent pathogen infection, by behavioral or physiological means (Møller et al. 1999). From a physiological point of view, perhaps the most efficient way of fighting parasites off is the immune system, designed to protect the host against a variety of pathogens, from virus to parasites. Most of the literature has been focused on the evolutionary meaning of the host capacity to develop an effective immune system to fight against pathogens. However, not all individuals have the same immune capacity to react to the same pathogen. Hamilton and Zuk (1982) linked the individual capacity of the hosts to fight against parasites within a sexual selection context. They suggested that sexual selection should favor individuals with genetic disease resistance by assessing the full expression of those secondary sexual traits that are dependent of individual's health. It implies that individuals with enhanced genetic capacity to fight against parasites are able to display their sexual traits at their best.

The expression of sexual characters is costly to produce and, according to Hamilton and Zuk (1982), such a cost can be caused by pathogen infection. In relation to carotenoid pigmented traits, parasite infection reduces the expression of these traits (Brawner et al. 2000; Martinez-Padilla et al. 2007), and the expression of carotenoid-pigmented traits may inform about males' ability to resist parasite infection (Lindstrom and Lundstrom 2000). From a physiological perspective, immune defense is the mechanism that allows hosts to fight against pathogen infection. A seminal paper by Blount et al. (2003) suggested that dietary carotenoid supply produced a parallel increase in carotenoid-dependent pigmentation and immune defense in zebra finches. Later on, it was proved that mounting an immune response is physiologically costly to produce in birds (Horak et al. 2000; Ots et al. 2001; but see Owen-Ashley et al.2004) resulting in negative fitness consequences (Marzal et al. 2007). Immune capacity is intimately linked to genetic quality (Hamilton and Zuk 1982), but interestingly carotenoids can boost immune defense (Møller et al. 2000; McGraw and Ardia 2003, 2007), resistance to parasite infection (Baeta et al. 2008) and mounting an immune response reduces the availability of carotenoids (McGraw and Ardia 2003; Alonso-Alvarez et al. 2004; Peters et al. 2004; Aguilera and Amat 2007; Pérez-Rodríguez et al. 2008). However, the inter-relations between immune capacity and carotenoid availability is more complex than initially though (Biard et al. 2009), deserving further research. Such cost on immune response, however, may constrain the expression of carotenoid-pigmented sexual signals. Thus, a physiological trade-off emerges because individuals should allocate their carotenoids to physiological functions (i.e. health or self maintenance) or to ornament pigmentation (Lozano 1994).
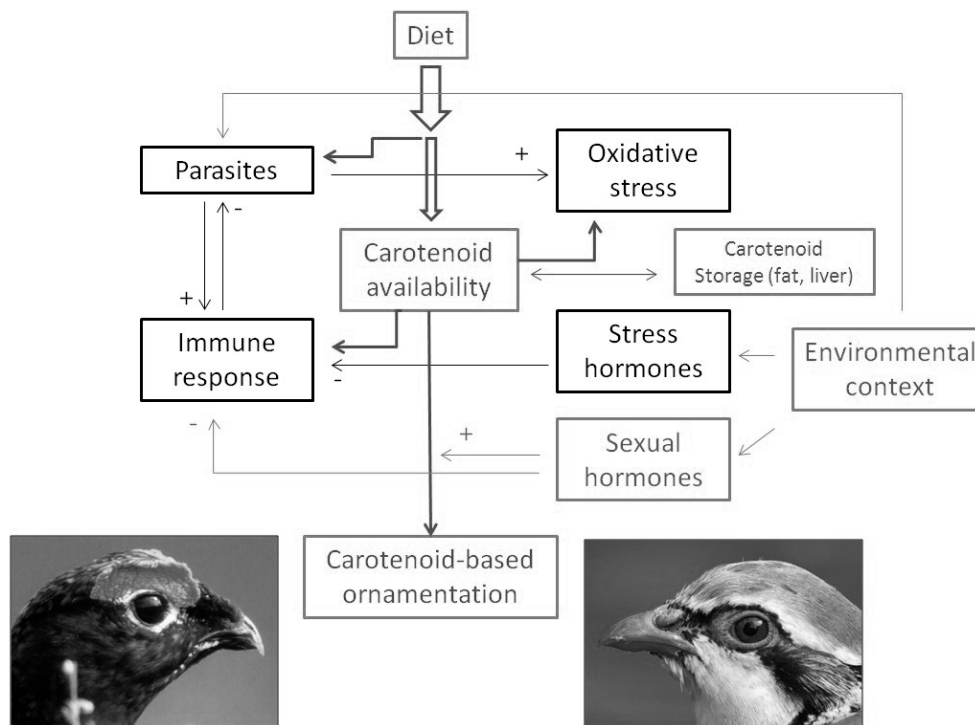
Figure 1. Summary of the pathways followed by carotenoid pigments from their aquisition through diet to their deposition in ornaments. Arrows indicate the inter-relations between four main components of an individual´s heatlh status (parasites, immune responses, oxidative stress and physiological stress), that can reduce the amount of available carotenoid pigments that are ultimately deposited in integuments and used for ornamental displays (combs for red grouse, below left, and red bill and eye rings for red-legged partridge, below right). These effects or allocation priorities are also modulated by sexual hormones (in particular testosterone) and by the environmental context (see text for further details).

Most sexual characters in males are modulated by hormones, and specifically by testosterone (Adkins-Regan 2005). This hormone plays a crucial role in sexual selection as it enhances the expression of secondary sexual traits, but also has immunosuppressive effects (Folstad and Karter 1992). Folstad and Karter (1992) suggested that testosterone may mediate the honesty of the sexual signaling based on an elegant refinement of the handicap principle (Zahavi 1975). They suggested that only high-quality individuals are able to afford the immunosuppressive effects of testosterone while showing the most elaborate sexual displays (Folstad and Karter 1992), what is known as the Immunocompetence Handicap Hypothesis (ICHH). This hypothesis predicts that testosterone and parasites act in opposite directions, such that testosterone increases the susceptibility to parasitism, and parasites constrain testosterone-dependent behavior or ornamentation in this case. Interestingly, testosterone may up-regulate the bioavailability of circulating carotenoids, that can be used for ornament pigmentation by one side or to counteract the immunosuppressive effects of testosterone on the other (Blas et al. 2006; Martinez-Padilla et al. 2010). This trade-off has been studied in red-legged partridges and red grouse (see below), but also in other species. Studies looking at allocation priorities of carotenoids are however still controversial and deserve further investigation. Peters et al. (2012) used a two-way experiment to simultaneously manipulate, testosterone and carotenoids, and found that testosterone increased circulating levels of carotenoids only in carotenoid-supplemented birds and that there was no effect of testosterone on plumage coloration. While several studies have explored the effects of immunity on carotenoid levels or carotenoid-pigmented traits, further experimental studies manipulating

testosterone and pathogen infection (or immune capacity) are needed for a deeper comprehension of the complex interactions involved (Figure 1).

These physiological trade-offs have been deeply studied in birds mainly in captive conditions and less intensively in free-living individuals. However, in natural conditions, individuals are under different environmental stressors that can influence how they resolve physiological trade-offs. This may explain some of the inconsistencies found between species (Owen-Ashley et al. 2004). In natural populations where environmental conditions are good, individuals may have a greater flexibility for allocating their carotenoids between ornamentation and immune defense. Conversely, in populations where environmental conditions are stressful (i.e. high parasite prevalence, low food abundance at population level) individuals can be more constrained in their allocation of carotenoids to ornamentation and immune defense, prioritizing immune defense instead of ornamental pigmentation. Examples of this idea are scarce in birds, but there is some support of this environmental-dependent resolution of carotenoid allocation trade-offs. In red grouse, individuals treated with testosterone increased their carotenoid levels only in populations where population density was low, and increases of testosterone were only positively associated with ornamental coloration in low-density population (Martinez-Padilla et al. 2010). The authors hypothesized that individuals living in low-density populations can store their carotenoids and use them when needed. In captive male zebra finches, the presence of females can affect the expression of carotenoid-based sexual traits under carotenoid-demanding conditions and lack of pathogen infection (Gautier et al. 2008). This suggests that the expression of the signal can be adaptive because it is modulated depending on the expected benefits in relation to the social environment individuals live in (Gautier et al. 2008).

The response of individuals to stressful situations is modulated by stress hormones (corticosterone in birds), which can act as mediators of sexual signal expression (Husak and Moore 2008). Free-living animals experience many stressors (e.g. weather, predators, parasites, social conflicts) that challenge their homeostasis (Romero 2004). A major adaptation is the hypothalamic–pituitary–adrenal axis, which releases glucocorticoids in response to stressors, allowing individuals to recover homeostasis in the best condition. However, chronic or inappropriate stress responses can exert deleterious effects, such as immunosuppression (Romero 2004). How an individual responds to or copes with stressors is likely a major determinant of its fitness, and ornaments could reveal how individuals cope with stressors. There is some evidence that the expression of carotenoid-based traits is associated with stress hormone levels, although the sign of the association may vary (e.g. Cote et al. 2010; Costantini et al. 2008; Loisseau et al. 2008; Bortolotti et al. 2009). The link between stress hormones and carotenoid-based traits may be mediated by their effects on immunity or oxidative stress (Romero 2004; Costantini et al. 2008).

Recently, it has been shown that choosing a parasite-free mate may reduce parasitism risk of the choosy partner (Martinez-Padilla et al. 2012). In fact, there in an asymmetry in such infection risk such as males have a higher increase of being parasitized than females. This may have implications for the ICHH (Folstad & Karter 1992), which was initially developed for males. If males mate with highly parasitized females, this extra cost may alter the resolution of the trade-off between investing in testosterone-dependent behavior and defense against parasites, and perhaps may also alter how carotenoids are allocated to carotenoid-pigmented sexual traits by males. Thus, parasite levels of female mates should be considered for a better comprehension of the effects of the ICHH.

CAROTENOID-BASED TRAITS AND OXIDATIVE STRESS IN BIRDS

Oxidative stress is the result of the imbalance between production of reactive oxigen species (ROS) and the antioxidant defences of the organism in favour of the former (Halliwell and Gutteridge 2007). ROS are highly reactive molecules that may damage (oxidize) important biomolecules such as DNA, lipids and proteins. Most endogenously produced ROS are normal by-products of aerobic metabolism. Apart from cell metabolism, another important source of ROS is the immune response. During an infection, the immune system cells generate ROS as a way to combat invading pathogens. Although ROS are produced as part of the killing mechanism, this may cause oxidative damage to the host organism, increasing the cost of immune response (Halliwell and Gutteridge 2007). During the last decades, 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 (Krinsky 1989; Rao and Agarwal 1999; Rao and Rao 2007; Halliwell and Gutteridge 2007).

The physiological trade-offs proposed by Lozano (1994) and von Schantz et al. (1999) to explain the honesty of carotenoid-based signals nourished from these studies highlighting the healthy properties of carotenoids in humans, resulting in an enthusiastic assumption of the "antioxidant role" hypothesis (Møller et al. 2000) (Figure 1). However, years later several studies questioned the key assumption of this ''antioxidant role'' hypothesis, claiming that carotenoids are not as powerful antioxidants as initially proposed (Hartley and Kennedy 2004; Isaksson and Anderson 2007; Costantini and Møller 2008; Isaksson et al. 2008). As an alternative way to connect oxidative stress and carotenoid-based ornamentation, despite a possible poor antioxidant capacity of these pigments, it has been suggested that carotenoids are particularly sensitive to be altered by ROS. This may result in a spurious relationship between carotenoid pigmentation and oxidative stress that is mediated by the quality of the antioxidant system (Hartley and Kennedy 2004).

The antioxidant properties of carotenoids have been a subject of controversy among ecophysiologist and behavioral ecologists during the last decade, and have been addressed by recent reviews (Costantini and Møller 2008; Catoni et al. 2008; Monaghan et al. 2009; Pérez-Rodríguez 2009; Svensson and Wong 2011). Although some criticisms regarding the antioxidant properties of carotenoids already exist in the medical literature (Rice-Evans et al. 1997;Young and Lowe 2001; Krinsky 2001), most of the controversy arises from the intrinsic differences between humans and birds, that impose restrictions to the extrapolation of the roles of carotenoids between them (Pérez-Rodríguez 2009). For instance, birds circulate different amounts and, most importantly, different types of carotenoids (birds circulate xantophylls whereas humans circulate mostly carotenes). This is crucial because, at least *in vitro*, carotenes show a higher ROS scavenging capacity than xanthophylls (Miller et al. 1996; Stahl et al. 1998). Also, there are important physiological differences between birds and humans that may affect the relative importance of carotenoids as antioxidants in both groups. For instance, birds, in contrast to mammals, are uricotelic and therefore maintain blood levels of uric acid three times higher than humans. Also, unlike humans, most bird species are able to synthesize vitamin C, a powerful antioxidant that interacts with carotenoids during redox reactions (Surai 2002). Both uric acid and vitamin C may deeply affect the ''antioxidant environment'' of an organism, which may in turn affect the action of carotenoids (Stahl and Sies 2003). In addition, susceptibility to oxidative stress may differ between taxa 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 (Stahl and Sies 2003).

For the reasons mentioned above, extrapolating the antioxidant role of carotenoids from humans or mammal models to birds seems questionable. Although some general patterns may

be conserved, elucidating the role of carotenoids can be validated using avian models. To date, experimental and correlational studies in birds have led to inconsistent results. This may be due, in many cases, to a simplistic conceptualization of the antioxidant system and redox reactions, to an incomplete asessment of the antioxidant defences and oxidative damage, or to experimental designs that do not allow to tease apart the antioxidant and immunostimulant roles of carotenoids (Pérez-Rodríguez 2009). However, a recent phylogenetically controlled meta-analysis performed on 88 different species of birds found that carotenoids are significantly positively related to plasma antioxidant capacity, but not significantly related to oxidative damage (Simons et al. 2012). The relationship between oxidative status and carotenoid-based ornamentation was positive, though not significant.

Further research is still required to clarify the importance of carotenoids as signals of oxidative stress in birds. 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 consider measures of antioxidant capacity and oxidative damage simultaneously, taking a dynamic approach and covering different tissues, in addition to blood parameters (Pérez-Rodríguez 2009, Simons et al. 2012). Finally, alternative pathways linking carotenoids to vital biochemical processes must be explored. In this sense, Hill and Johnson (2012) recently proposed a biochemical model for the regulation of ornamental coloration based on interdependencies of carotenoid and retinoid biochemistry. This model proposes that vitamin A regulatory mechanisms, redox systems, and carotenoid pigmentation pathways link carotenoid coloration to oxidative status and other aspects of host performance, such as immunocompetence. Based on complex enzymatic cycles and pathways, this model offers a novel window to study the physiological basis of the honesty of carotenoid-based signals, in birds as well as in other taxa.

CAROTENOID-BASED ORNAMENTS AS SIGNALS OF QUALITY IN THE RED-LEGGED PARTRIDGE (*ALECTORIS RUFA*)

The red-legged partridge (*Alectoris rufa*) is a medium sized game bird belonging to the family Phasianidae, endemic to the western Mediterranean where its natural distribution encompasses France, NW Italy and the Iberian Peninsula including the Mediterranean islands of Corsica, Elba and the Balearics. It inhabits areas of a wide variety of land uses, although it shows the highest densities in the centre-south of Spain, and is least abundant along the Mediterranean coast (Blanco-Aguiar et al. 2003).

Both sexes spend the winter in sedentary flocks. However, from January-February, males exhibit territorial behavior and perform visual and vocal displays to attract females. The species is socially monogamous. Mating takes place between February and April, and egg laying between April and early June (Cramp and Simmons 1980). Females typically lay and incubate one clutch of 6-15 eggs, but in some cases (up to 50% of pairs in good years) they lay a second clutch that is entirely incubated by the male (Green 1984; Casas et al. 2009). During the first weeks after hatching, partridge chicks feed on insects, but they soon move to a diet based on seeds and green plants that they will maintain throughout their lives.

Figure 2. Two images of a male red-legged partridge showing its melanin based plumage pattern and its carotenoid-pigmented beak, legs and eye rings. Photo credits: Rafael Palomo.
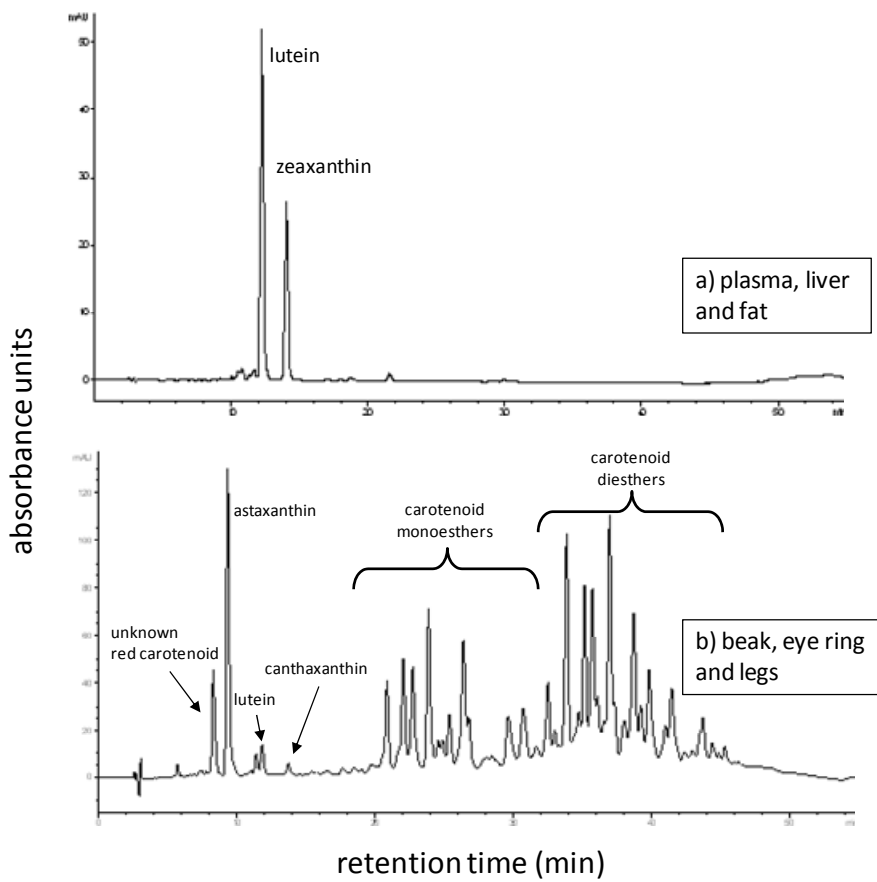

Figure 3. Illustrative High Performance Liquid Cromatograms showing the carotenoid composition of a) plasma, liver and fat and b) any of the red traits (beak, legs and eye rings) of the red-legged partridge. Courtesy of Esther García de Blas.

The red-legged partridge shows a slight sexual size dimorphism (males weigh ca. 480 grams, whereas females are 70 grams lighter on average; overall body size show marked variations across the area of distribution). However, males and females show the same plumage pattern (Figure 2), characterized by a black spotted bib and a barred flank feather tract. The back is olive-green, whereas the chest and the belly are grey and orange, respectively. Carotenoids are absent from this plumage pattern, which is pigmented by pheomelanin and eumelanin instead. However, both sexes show conspicuous and carotenoid-based red legs, beaks and eye rings that significantly differ in color intensity between sexes (Villafuerte and Negro 1998; Pérez-Rodríguez 2008).

The red coloration of the beak, legs and eye rings of the red-legged partridge results from the accumulation of red carotenoids in the ramphoteca (beak), podotheca (leg) and epidermis (eye ring) of the bird. High Performance Liquid Chromatography analyses have revealed that the main carotenoids in all three integuments are astaxanthin and a still unknown carotenoid whose structure appears similar to that of papilioerythrinone (García-de Blas et al. 2011; García-de Blas et al. *in press* - Figure 3). Also, small amounts of lutein and canthaxanthin are found in all three ornaments. Interestingly, both astaxanthin and the unknown red carotenoid are mostly (>80%) in esterified forms (mono- and di-esters of carotenoid). Lutein and cantaxanthin, however, did not produce esterified compounds. Importantly, there are only two carotenoids in the plasma and internal tissues (liver, fat) of the red-legged partridge: lutein and zeaxanthin (Figure 3) that are acquired through diet. This implies that the red carotenoids found in ornamental traits (and their esters) are synthesized within integuments from these two dietary carotenoids.

Carotenoid esters have never been found in feathers of any species (McGraw 2006). However, it may be the rule in living tissues of birds (Czeczuga 1979; Casagrande et al. 2011) and other taxa, like fishes or crustaceans (e.g. Wade et al. 2008; Pike et al. 2011). The role of carotenoid esterification in these tissues is still unknown. In the red legged partridge, carotenoid esters where better predictors of integument redness than total carotenoids in the tissue (although the latter still correlates well with overall ornament coloration). It is therefore likely that pigment esterification could play a role in carotenoid stabilization or mobilization to/from living tissues for signaling purposes. This hypothesis, however, requires further experimental support.

There is a slight but significant sexual dimorphism in carotenoid-based coloration in the red-legged partridge, males showing redder eye rings and beaks than females (Pérez-Rodríguez and Viñuela 2008; Pérez-Rodríguez 2008). Also, the proportion of the eye ring skin pigmented by carotenoids (eye ring pigmentation, hereafter) is greater in males than in females (Pérez-Rodríguez and Viñuela 2008; Pérez-Rodríguez 2008). As many other carotenoid-based ornaments, the legs, eye rings and beak of the red-legged partridge show a certain degree of reflectance in the ultraviolet range of the visual spectrum. As found in the fleshy ornaments of grouse (Mougeot et al. 2007a), ultraviolet reflectance of partridge ornaments seem to increase when carotenoids deposited in the ornament are scarce and the underlying dermis layers (eye ring) or the outermost keratinized parts of the integument contribute relatively more to ornament reflectance (Pérez-Rodríguez 2008). One direct consequence of this is that ornament redness and relative UV reflectance are negatively related, and the higher the carotenoid concentration in the integument, the higher its relative red saturation and hue, and the lower its ultraviolet reflectance is (Pérez-Rodríguez 2008).

One important feature of carotenoid-pigmented living tissues is that their color can be dynamically adjusted according to the condition or status of the individual. In the red-legged partridge, eye ring and beak redness mirrored seasonal variations in circulating carotenoids. This results in higher expression of carotenoid-based ornamentation coinciding with the end of the mating season and the start of laying, which is consistent with a signaling role of these

traits in sexual selection scenarios. These changes could be partly attributed to the action of androgens, which increase carotenoid absorption and regulate their mobilization to ornamental traits in this species (Blas et al. 2006; Alonso-Álvarez et al. 2008, 2009). However, besides this dynamism in coloration, red color expression relative to others in the study population was consistent across the breeding season and between consecutive years, thus indicating that carotenoid-based traits in the red-legged partridge accurately reflect consistent differences in individual quality (Pérez-Rodríguez 2008).

Further evidences are also consistent with a signaling role of carotenoid-based traits in the red-legged partridge. For instance, when redness of the beaks and eye rings of males is artificially enhanced by means of paint, their females lay earlier and produce more eggs than those mated with control (un-manipulated) males (Alonso-Alvarez et al. 2012). Therefore, females respond to male coloration and adjust their allocation to current reproduction accordingly (Andersson 1994). Field data also support a signaling role of carotenoid-based coloration among partridges, as males and females mate assortatively according to beak coloration in the wild (Mougeot et al., *unpublished data*), which is may be considered as evidence of mutual mate choice.

Carotenoid-based ornamentation in the red-legged partridge is related to several different indicators of health status. Eye ring and beak redness are related to individual condition and to the degree of physiological stress, estimated using the relative abundance of heterophils in the blood as a proxy (Pérez-Rodríguez and Viñuela 2008; Mougeot et al. 2009a). Experimental manipulations of body condition resulted in rapid reductions of eye ring coloration, but were not mirrored by short-term changes in beak redness. This suggests that different ornaments reflect changes in body condition but at different speeds or intensities: eye ring, a fleshy ornament, appears to respond more rapidly to changes in the nutritional status than the bill, which is a keratinized structure (Pérez-Rodríguez and Viñuela 2008). Also, individuals with higher abundance of coccidia (an intestinal parasite) showed lower levels of circulating carotenoids, which resulted in lower eye ring redness (Mougeot et al. 2009a). Coccidia are known to affect carotenoid absorption, mainly because of the damage caused to the intestinal mucosa (Allen 1987). In addition, the immune response against parasite infection, which is often associated to an increase of ROS production (Halliwell and Gutteridge 2007), may also contribute to the negative effect of coccidia on circulating carotenoid levels and carotenoid-based ornamentation.

Circulating carotenoids and carotenoid-based coloration of eye rings and beak are also positively related to the cell-mediated immune responsiveness, measured as the skin swelling response to the intra-dermical injection of a plant lectin, phytohaemagluttinin (Pérez-Rodríguez et al. 2008; Mougeot et al. 2009a). Such an immune challenge also induced a 13% decrease in circulating carotenoids within 24 hours although that decrease did not affect carotenoid-based coloration, probably because of the short term effect of the T-cell-mediated immune response. However, these results highlight a link between immunity and carotenoids, which are consumed during immune responses.

Besides the links among carotenoids, immune response and oxidative stress, the above mentioned decrease in circulating carotenoids in partridges exposed to an immune challenge did not appear to be strongly associated with oxidative stress (Pérez-Rodríguez et al. 2008). In fact, T-cell-mediated immune challenge did not affect oxidative stress markers (lipid peroxidation, antioxidant capacity). Also, neither circulating carotenoids nor carotenoid-based coloration were related to oxidative stress. However, individual changes in oxidative damage during immune response were predicted by individual coloration, suggesting that carotenoid-based traits in this species may foresee an individual´s ability to resist oxidative stress (Pérez-Rodríguez et al. 2010). Finally, oxidative stress affects carotenoid circulation and deposition into red ornaments: when exposed to low doses of a source of ROS (diquat) during four

months, red legged partridges showed lower levels of circulating carotenoids and paler carotenoid-based ornaments (Alonso-Alvarez and Galván 2011). This supports the hypothesis that carotenoids are sensitive to oxidative stress, which is reflected by the ornaments based on these pigments, which can be considered as an adequate proxy of oxidative status in this species.
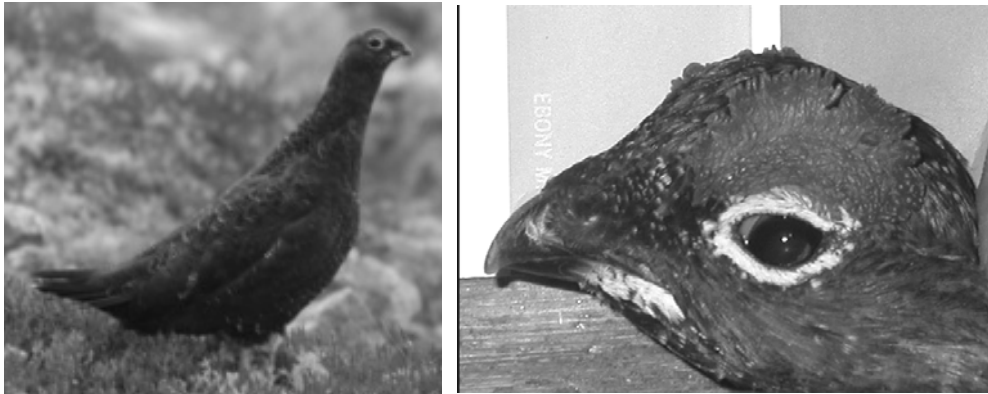

Figure 4. Two images of a male red grouse displaying its supraorbital red com. Photo credits: Gary R. Bortolotti and Jesús Martínez Padilla.

CAROTENOID-BASED ORNAMENTS AS SIGNALS OF QUALITY IN THE RED GROUSE (*LAGOPUS LAGOPUS SCOTICUS*)

Grouse (Tetraonidae family) are medium sized game birds of the northern hemisphere, living in arctic, boreal and temperate region (Watson and Moss 2008). Grouse display bright yellow red supra-orbital combs, a main sexual ornament that is pigmented by carotenoid pigments (Egeland et al. 1993; Mougeot et al. 2007a; Siitari et al. 2007) (Figure 4). Grouse combs reflect in the ultraviolet (UV), which is not visible to humans but is likely to be visible to grouse (Mougeot et al. 2005). Comb UV reflectance decreases with increasing comb size and redness, and is mainly a property of the un-pigmented skin, with the carotenoid pigments deposited in the epidermis to increase the orange-red coloration acting as a mask that reduces skin UV reflectance (Mougeot et al. 2007a). Combs are displayed by both sexes, although they are typically larger and brighter in males (Mougeot and Redpath 2004; Martinez-Padilla et al. 2011) (Figure 4). Both the size and carotenoid-coloration of combs are testosterone-dependent; in red grouse, for example, males circulating more testosterone display larger and redder (Mougeot et al. 2005; Martinez-Padilla et al. 2010). Moreover, males with higher testosterone levels are typically dominant and more aggressive, and have higher mating success, suggesting that comb size and coloration functions in intra- and inter-sexual selection processes (Redpath et al. 2006b).

A main parasite of red grouse is the nematode *Trichostrongylus tenuis*, which as well known negative effects on condition, reproduction and survival (Hudson 1986; Hudson et al. 1992; Redpath et al. 2006a). *T. tenuis* parasite abundance can be easily measured (Seivwright et al. 2004) and manipulated in free-living red-grouse, by means of parasite purging (dosing with anthelminthic to reduce parasite abundance) or by means of challenges with parasite infective larvae, in order to increase parasite abundance (e.g. (Mougeot et al. 2005; Seivwright et al. 2005; Martinez-Padilla et al. 2007; Mougeot et al. 2007b; Mougeot et al. 2009; Mougeot et al. 2010a; Mougeot et al. 2010b). This has allowed to test the effects of *T. tenuis* parasites on carotenoid-based ornamentation (comb redness), and to demonstrate that males with more parasites, which are typically in poorer condition, circulate fewer

carotenoids, and have less colored combs (Martinez-Padilla et al. 2007; Mougeot et al. 2007b; Martinez-Padilla et al. 2010; Mougeot et al. 2010a). There are several, non-exclusive, mechanisms that may explain this effect of parasites on carotenoid-based ornamentation. First, intestinal parasites may compete with the host for the absorption of carotenoids acquired through diet, and birds with abundant parasites may have a reduced absorption, and fewer carotenoid pigments available overall. Second, hosts have to mount an immune response to the parasite infection, and given that carotenoids have immune-stimulant properties, those red grouse with more parasites would have fewer carotenoids available for boosting their immune response, and might be forced to use available pigments for this to the detriment of using them for displaying redder combs (Mougeot et al. 2007b). Male red grouse with redder combs have a greater ability to mount a T-cell-mediated immune response than males with duller combs (Mougeot 2008), and therefore might advertise that they are better able to respond to a new immunological challenge. This might be because they are overall healthier (i.e. have fewer parasites) and therefore more able to use available carotenoid to respond to immune challenges. Third, parasites have been shown to cause oxidative stress, i.e. to increase levels of oxidative damage and reduce the availability of circulating antioxidant defenses; (Mougeot et al. 2009b; Mougeot et al. 2010a). This would imply that red grouse with more parasites might need to use more of their available carotenoid pigments to reduce oxidative stress and, as a result, would have fewer carotenoids available for increasing ornamental coloration. In that respect, experiments have shown that reductions in *T. tenuis* parasite abundance increase carotenoid-based ornamentation, proportionally to the change in oxidative balance. Specifically, following parasite purging, those males that increased their comb redness the most, were those that increased antioxidant defenses and reduced oxidative damage most, (Mougeot et al. 2010a). This suggests that the effects that parasites have on carotenoid-based ornaments might be mediated by oxidative stress. Finally, there is evidence for stress hormones, specifically corticosterone, to also modulate the effects of *T. tenuis* parasites on comb redness. After parasite purging, those males that experienced less physiological stress overall (measured as the overall amount of corticosterone deposited in feathers grown during experiment), were able to increase their comb redness the most (Mougeot et al. 2010b).

CONCLUSION

Carotenoid-based ornaments are a paradigm among sexual signals in animals, especially in birds. Honest signalling theory predicts that the production and maintenance of reliable signals must entail costs (or limitations) for the bearer. Carotenoids seem to be a limited resource for most species, which implies that the expression of carotenoid-based colorations will be higher in those individuals with better foraging capacity. This implies that carotenoid-based ornaments may serve as signals of body condition and foraging ability. In addition, potential links between carotenoids and key physiological processes, like immune responses and oxidative stress, may result in new pathways for the honesty of these traits.

In the red-legged partridge, the red coloration of the beak, eye rings and legs is based on carotenoid pigments (mostly astaxanthin plus another red carotenoid still unidentified) that are metabolized by the bird from dietary carotenoids (lutein and zeaxanthin). These changes take place at the integuments, and >80% of deposited carotenoids are esterified. Despite the fact that mate choice experiments have not been performed yet, there is evidence supporting a signaling role of these traits in this species: individuals mate assortatively according to beak redness in the wild, and beak and eye ring coloration are maximized during the mating season. Furthermore, experimental enhancement of male redness resulted in higher breeding

effort by females, who seem to perceive trait expression as a signal of mate quality. In fact, beak and eye ring redness are dynamic traits related to individual nutritional and health status. The redness of these ornaments is related to circulating carotenoids, and both are positively related to T-cell-mediated immunocompetence and negatively to intestinal parasite (i.e. coccidia) infection levels. Although there are no sound evidences that carotenoids may exert a powerful antioxidant action in this species yet, they are sensitive to increased oxidative stress, which reduces their circulating levels and reduces ornament redness. Taken altogether, these results support the hypothesis that carotenoid-based ornaments function as honest signals of health (condition, immunocompetence, parasite infection and oxidative status) in the red-legged partridge.

As in may other tetraonid species, the red grouse exhibit bright red supraorbital combs pigmented by carotenoids. These ornaments are present in both sexes, but are more developed in males than in females, and their size and color are maximized during the mating season, and these ornements likely play a role both in inter- and intra-sexual selection processes. Comb redness is possitively associated with body condition and T-cell-mediated immunity, and negatively affected by coccidia and *T. tenuis* infection, a main intestinal nematode parasite. In fact, the negative impact of *T. tenuis* on red grouse combs has been repeatedly demonstrated in several field experiments. This negative effect of *T. tenuis* on comb redness appears to be mediated, at least in part, by parasite effects on oxidative balance, that seems to reduce the amount of bioavailable carotenoids for physiological functions. Carotenoid-allocation to ornaments is modulated by testosterone levels, though this effect depends on the social or population context. Therefore, as in the red-legged partridge, these results also support the hypothesis that carotenoid-based ornamentation advertises an individual´s overall health status (nutritional status, parasite levels, immunocompetence) and that their expression could be mediated by the environmental context individuals live in..

There are still many relevant questions that deserve further research. In the specific case of the red-legged partridge, field experiments that simultaneously manipulate hormone levels and oxidative stress would be necessary to validate the above mentioned results obtained under captive conditions. This is relevant because wild partridges circulate greater amounts of carotenoids than captive ones, and also display redder ornaments (Garcia-de Blas et al. *in press*). Also, experiments combining carotenoid supplementation and oxidative challenges are required to fully assess the antioxidant function of carotenoids. The link between carotenoids and humoral immune responses, and between carotenoids and parasites other than coccidia, are necessary to obtain a more complete view of the conections between these pigments and the immune system. In the red grouse, experimental designs directly manipulating oxidative stress independently of parasite and testosterone levels are also required to fully assess the importance of this factor as a regulator of carotenoid-based ornamentation.

Beyond these two model species, our understanding of the function of carotenoid-based signals would benefit from biochemical studies on the transformation of ingested carotenoids (at the enzymatic and genetic level) and on the pathways involved in all steps of carotenoid use and metabolism. Studies using radio-labeled carotenoids would be very helpful in that respect. Furthermore, studies performed to date have paid a disproportionate attention to plasma carotenoids, whereas other carotenoids in tissues have been neglected. A complete understanding of the roles of these molecules in the organism, particularly regarding their controversial antioxidant roles, would require an exploration of their effects in other tissues, and in phases of the living cycle other than adulthood. Also, opening the mind to new possible and testable pathways linking carotenoid-based coloration to key physiological proceses (such as vitamin A metabolim, for instance; Hill and Johnstone 2012) will result in a better comprehension of the evolution of these traits as indicators of health. Finally, a still pending issue is to address the connection between ecological and physiological factors

affecting the allocation of carotenoids (among other resources) to ornament expression. Environmental variables like age- and sex-ratios, population density, climatic conditions, resource availability, etc. are expected to strongly influence individual trade-offs. Establishing the connections between environmental factors and physiological trade-offs remains a major challenge for evolutionary ecologists.

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