

Carotenoid-based signals in behavioural ecology: a review

P.A. Svensson¹⁾ & B.B.M. Wong

(School of Biological Sciences, Monash University, Clayton, VIC 3800, Australia)

(Accepted: 26 November 2010)

Summary

Carotenoids are among the most prevalent pigments used in animal signals and are also important for a range of physiological functions. These concomitant roles have made carotenoid-based signals a popular topic in behavioural ecology while also causing confusion and controversy. After a thorough background, we review the many pitfalls, caveats and seemingly contradictory conclusions that can result from not fully appreciating the complex nature of carotenoid function. Current controversies may be resolved through a more careful regard of this complexity, and of the immense taxonomic variability of carotenoid metabolism. Studies investigating the physiological trade-offs between ornamental and physiological uses of carotenoids have yielded inconsistent results. However, in many studies, homeostatic regulation of immune and antioxidant systems may have obscured the effects of carotenoid supplementation. We highlight how carefully designed experiments can overcome such complications. There is also a need to investigate factors other than physiological trade-offs (such as predation risk and social interactions) as these, too, may shape the expression of carotenoid-based signals. Moreover, the processes limiting signal expression individuals are likely different from those operating over evolutionary time-scales. Future research should give greater attention to carotenoid pigmentation outside the area of sexual selection, and to taxa other than fishes and birds.

Keywords: signal honesty, sparing hypothesis, protection hypothesis, trade-off, handicap, homeostasis.

¹⁾ Corresponding author's e-mail address: andreas.svensson@monash.edu

1. Introduction

Carotenoid-based ornaments account for some of the most spectacular visual signals found in the animal kingdom, from the fiery red beaks of zebra finches (e.g., Collins & ten Cate, 1996) to the colourful dewlaps of anolis lizards (e.g., Macedonia et al., 2000). Interest in carotenoid-based signals originated with John Endler's classic work on guppies in the early 1980s (Endler, 1980, 1983) and have since expanded considerably, especially in recent years, due to advances in our understanding of signalling theory, biochemistry, physiology and immunology. Carotenoids, in this regard, are important, not only because of their role as some of the most ubiquitous ornamental pigments found in nature (McGraw, 2006b), but also because of their various physiological functions (Britton, 2008). The dual roles of carotenoids in signalling and physiology have made them a favoured subject in studies of animal signals (McGraw, 2006b; Blount & McGraw, 2008). While the different functions have certainly helped fuel the wealth of research in this field, they have also been the source of much confusion. Indeed, theories explaining the function and evolution of carotenoid-based ornaments have only recently started to receive the rigorous and critical testing required.

The field of carotenoid-based signals has witnessed major paradigmatic shifts in how patterns and relationships are interpreted. Many aspects of this development (and the ensuing controversies) have been covered by existing reviews (e.g., McGraw, 2006b; Peters, 2007; Blount & McGraw, 2008; Costantini & Møller, 2008; Perez-Rodriguez, 2009; Vinkler & Albrecht, 2010). These recent papers, however, are quite varied, both in terms of topic and breadth. More often than not, they also tend to be targeted towards already established researchers within the field. What is critically lacking is a review geared towards behavioural ecologists that seeks to consolidate a vast and bewildering body of literature whilst, at the same time, remain sensitive to readers who may not have the depth of knowledge often necessary to navigate the field. The main focus of this review will be on ideas concerning how the physiological functions of carotenoids relate to signal content (e.g., individual 'quality'), and on how the honesty of carotenoid-based ornaments may be maintained. To put the recent developments in context, we believe that it is vital to provide a thorough background and context to guide new researchers. The first section, therefore, introduces key concepts in carotenoid chemistry, as well as briefly summarizes signalling theory and

how carotenoid-based signals relate to the broader literature on signals and signal honesty. From there, we evaluate recent suggestions that ornamental carotenoids are largely unimportant as antioxidants, and outline how properly conceived experiments can reveal underlying mechanisms whilst, at the same time, avoid some of the past mistakes that have confounded earlier empirical work. We also highlight some common pitfalls and caveats that have hampered (and in some cases continue to hamper) stringent deductions in this field. The final sections deal with unresolved issues that have either been neglected or deserve closer attention, as well as specific challenges that, in our opinion, offer considerable promise for future research.

1.1. Biochemical background

Carotenoid-based signals are becoming an increasingly popular study subject among behavioural ecologists. However, to successfully address the evolutionary aspects of carotenoid ornamentation, it is important to first understand some of the underlying principles of carotenoid biochemistry and function. In this section we will introduce readers to basic carotenoid chemistry and their role in oxidant and immunological homeostasis.

1.1.1. Carotenoid chemistry

Carotenoids are fat soluble hydrocarbons that can be biosynthesized only by photosynthetic organisms and certain bacteria and fungi (Goodwin, 1984). Due to their inability to biosynthesize carotenoids *de novo*, animals must generally rely on diet for their supply of carotenoids, although many are capable of structural modification of ingested carotenoids (Fehl et al., 2005). Presently, more than 750 known natural carotenoids have been described (Britton et al., 2004). They are broadly divided into carotenes, which are composed of only carbon and hydrogen, and xanthophylls, which are oxygenated derivatives.

The molecular structure of carotenoids can be quite varied, but most are tetraterpenoids with a carbon backbone consisting of 40 carbon atoms. All carotenoids share a common feature: the chromophore, which is a system of alternating single and double bonds in the central part of the molecule (Figure 1). This conjugated structure makes carotenoids effective in absorbing the energy from light at wavelengths from 400–500 nm (blue-green) (Bjørnland, 1997) which makes carotenoids appear yellow, orange or red to human

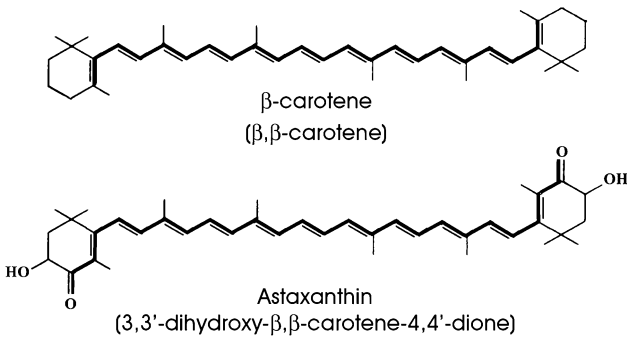


Figure 1. Example of two common carotenoids in animals: β -carotene (a carotene), and astaxanthin (a xanthophyll). The chromophores are indicated in bold.

eyes. In plants, carotenoids function as photosynthetic ‘antennae’, transferring the energy of blue and green wavelengths to chlorophyll, thereby expanding the spectrum that can be used for photosynthesis and increasing the light trapping efficiency of plants (Cogdell, 1985; Siefermann-Harms, 1985). When leaves of deciduous trees senesce in the fall, the green chlorophyll is degraded exposing the red and yellow colours of the carotenoids. The chromophore is also responsible for the antioxidant function of carotenoids. Specifically, the conjugated structure allows delocalization and stabilization of the energy contained in potentially damaging high energy molecules, such as radicals and other reactive oxidative metabolites.

Carotenoids may occur in pure form, or in association with other compounds. In animal skin, for example, carotenoids are often esterified (i.e., joined with fatty acids) (e.g., Glover et al., 1952), while in bird plumage, carotenoids are bound to keratin (Brush, 1990). Carotenoids may also form chemical complexes with proteins (forming carotenoproteins), which may dramatically affect their colour, producing purple, blue or even green hues (Zagalsky et al., 1970). One example is the blue-coloured exoskeleton of lobsters (*Homarus* sp.), which becomes red after boiling, as the heat denatures the carotenoprotein revealing the pure colour of the carotenoid astaxanthin (Cianci et al., 2002). In eggs, carotenoids are commonly incorporated in their free form, dissolved in the lipids. Carotenoids are transported around in lymph and blood by different lipoproteins (Parker, 1996; Lubzens et al., 2003). In other tissues, they often reside within the phospholipid membranes of cells. On a subcellular level, carotenoids appear to be concentrated in the membranes of organelles with the highest metabolic rates, primarily mito-

chondria (Chew & Park, 2004). In birds, carotenoids can also accumulate inside lipid droplets of the skin cell (keratinocyte) cytosol (Vanhoutteghem et al., 2004) and in the retina (Rahman et al., 2010). Importantly, carotenoids are not distributed evenly, nor are their relative concentrations uniform, between tissue types (Surai et al., 2001b). As we shall see below, this has important implications for quantifying carotenoids in animal tissue.

Carotenoids interact with many other bio-molecules *in vivo*, including proteins and lipids (Britton, 1995). Although they reside inside the cell membranes, their structure and polarity will affect their precise orientation and function within the membrane. For instance, carotenes, which are highly non-polar, reside deep in the hydrophobic core, while xanthophylls, with their polar end-groups, tend to be positioned across the membrane (Young & Lowe, 2001). Similarly, carotenoids of different polarity are not only transported by different types of lipoprotein, but their polarity also influences their position within the lipoprotein (Parker, 1996). The chemical structure, therefore, affects which types of molecules carotenoids come into contact with. Accordingly, important functional differences between carotenoids *in vivo* might not be detectable *in vitro*. Mechanisms for uptake, conversion and utilization can be very specific (Parker, 1996), even to isomers of the same carotenoid (e.g., Østerlie et al., 1999). This is partly because compounds, such as enzymes and lipoproteins, can be sensitive to the precise molecular structure of the carotenoid. Consequently, different tissue and cell types may respond to dietary carotenoids in different, possibly even opposing, ways (Chew & Park, 2004).

1.1.2. *Oxidants and antioxidants*

Oxidants are reactive molecules that may damage vital bio-molecules such as proteins, lipids and DNA (Handelman, 1996). They include singlet oxygen ($^1\text{O}_2$), hydroperoxides and molecules with unpaired electrons called radicals (formerly 'free radicals', Eberhardt, 2000). These high energy oxygen-containing oxidants are collectively called active, or reactive, oxygen species (ROS, Handelman, 1996). As suggested by Eberhardt (2000), we will instead use the term reactive oxygen metabolites (ROM) throughout this review, because it also encompasses compounds like nitric oxide. The formation of ROMs is caused by normal aerobic metabolism, but may also arise in numerous other processes, such as exposure to light. Notably, the powerful properties of ROMs can be put to good use, as seen, for example, when lysoso-

mal enzymes destroy pathogens (von Schantz et al., 1999, see section 1.1.6, Carotenoids and immune response).

Antioxidants are molecules that prevent or interrupt the detrimental chain reactions initiated by ROMs. An antioxidant is defined as “any substance that delays, prevents or removes oxidative damage to a target molecule” (Halliwell & Gutteridge, 2007, p. 81). Excited molecules, such as singlet oxygen, can be quenched (i.e., physically returned to their low-energy ground state) by the antioxidant (Handelman, 1996). Antioxidants may also chemically scavenge (i.e., reduce the concentration of) radicals by converting them to a more stable product while, themselves, becoming relatively harmless antioxidant radicals (Miki, 1991; Edge et al., 1997).

1.1.3. Oxidant homeostasis

The generation of ROMs is a part of normal metabolism, and a necessary consequence of living in an oxygen rich environment (Dowling & Simmons, 2009). Physiological homeostasis between ROMs and antioxidants is maintained through several systems, for example by up- and down-regulation of antioxidant enzymes (Finkel & Holbrook, 2000). Both endogenous and exogenous (dietary) antioxidants are instrumental in maintaining oxidative homeostasis (Schwedhelm et al., 2003). A very important consequence of this is that moderate alterations of the oxidant balance, for example through dietary antioxidant supplementation or mild infection may be homeostatically buffered, masking any changes taking place. Instead, the effects may only be detectable over time, or in other, seemingly unrelated, systems (e.g., reduced colouration caused by reallocation of antioxidant pigments, Alonso-Alvarez et al., 2008). Importantly, the strategies of such homeostatic adjustments may differ between individuals (Alonso-Alvarez et al., 2004, 2008). Animals have an arsenal of antioxidants at their disposal, and if one is in excess, it may lead to the sparing of another, without changing the overall balance (von Schantz et al., 1999, see section 2.2.2, Sparing). The term oxidative stress refers to “a disturbance in the pro-oxidant–antioxidant balance in favour of the former, leading to potential damage” (Sies, 1991, p. 15). Thus, if the homeostatic balance is overwhelmed by an excess of ROMs, it will result in oxidative stress, and may lead to damages of cellular constituents (Oakes & Van der Kraak, 2003). Conversely, if antioxidant levels become too high, normal cell functions may actually be impaired, because

ROMs are essential intermediates in many cellular systems (Finkel & Holbrook, 2000). As we shall see below (section Protection and sparing), homeostatic regulation may effectively mask short-term effects of experimental manipulation, making results difficult to interpret. However, even if elevated oxidative stress has limited immediate effects, it may still have longer-term implications, such as increased rate of senescence or reduced growth, reproduction and survival (Monaghan et al., 2009).

1.1.4. Carotenoids as antioxidants

It has been shown that carotenoids are powerful antioxidants, both in vitro (Burton, 1989; Krinsky, 1989; Miller et al., 1996; Martin et al., 1999) and in vivo (Miki, 1991; Krinsky, 1993; Britton, 1995; Shimidzu et al., 1996; Surai et al., 2001a), and can counteract several types of oxidants. The conjugated structure of the chromophore allows delocalization and stabilization of the energy contained in molecules with unpaired electrons, such as radicals (Britton, 1995). In plants, they quench triplet chlorophyll which is a harmful oxidant produced during photosynthesis (Edge et al., 1997). Carotenoids have also been shown to quench singlet oxygen ($^1\text{O}_2$) and scavenge radicals (Krinsky, 1989; Handelman, 1996; Edge et al., 1997), making them useful as antioxidants also in animals.

By quenching singlet oxygen, carotenoids can prevent the very formation of radicals. Upon contact, the carotenoid is excited to a triplet state, but quickly returns to the normal state, dissipating the excess energy as heat (Handelman, 1996). In this physical reaction, carotenoids work as catalysts and are not depleted (Edge et al., 1997). Studies have shown that one molecule of β -carotene is able to quench >100 000 molecules of singlet oxygen without being destroyed (Handelman, 1996). Xanthophylls, such as astaxanthin, are suggested to have an even higher quenching capacity than both β -carotene and vitamin E (Miki, 1991). Importantly, carotenoids can also be efficient at quenching singlet oxygen at the low concentrations found in vivo (Krinsky, 1989; Handelman, 1996).

The chemical reactions between carotenoids and already formed radicals are more complicated and less known. Most likely, the 'odd' electron of the radical is transferred to the carotenoids thereby creating a carotenoid radical (Edge et al., 1997), which then is repaired by other antioxidants (see next section). Carotenoids are also effective inhibitors of lipid peroxidation (Krinsky, 1989), a process where ROM degrades lipids, especially in cell

membranes, causing cell damage. In fact, most direct support of the antioxidant effect of carotenoid *in vivo* comes from evaluations of lipid peroxidation (Krinsky, 1993). For a recent review of the antioxidant role of carotenoids in evolutionary biology, see Pérez-Rodríguez (2009).

1.1.5. Carotenoids as parts of the antioxidant arsenal

It is important to emphasize that carotenoids are far from the only antioxidants available to organisms. All animals rely on a complex antioxidant network which includes endogenously produced enzymes and low-molecular weight compounds, but also exogenously obtained compounds like vitamins, carotenoids and other food-derived antioxidants. Enzymes such as superoxide dismutase, vitamins (especially E and C), melatonins, uric acid, flavonoids, complexing proteins and peptides, may all be used to combat ROMs (Eberhardt, 2000). Due to the homeostatic nature of antioxidant defence, a lack of one of these compounds can, at least partially, be compensated for by increased usage of another (Hörak et al., 2007, see section 2.2, Protection and sparing). In fact, one of the best described function of carotenoids *in vivo* is their ability to modulate the endogenous levels of other antioxidants (Krinsky, 1993). Although antioxidants of similar solubility may compete at the site of absorption, they are likely to interact positively following uptake (Catoni et al., 2008). For instance, carotenoids are known to work together with other antioxidants such as vitamin E (tocopherol) and vitamin C (ascorbate) *in vivo*. In particular, carotenoids can repair vitamin E radicals (Costantini, 2008), and if a carotenoid radical is formed, it may in turn be repaired by vitamin C (Young & Lowe, 2001). Consequently, when both carotenoids and vitamin E are present, the combined antioxidant activity is greater than the sum of their individual contributions (Palozza, 1998; Amar et al., 2001; Catoni et al., 2008). As we shall see below (section 2.2, Protection and sparing), effects of carotenoids can be highly contingent on which other antioxidants are present in the same tissue (Catoni et al., 2008). Due to antioxidant synergisms, the actions of carotenoids may differ greatly between studies done *in vitro* and *in vivo*. Even carotenoids with similar structures may have vastly different effects *in vivo*, such as, for example, highly differing antioxidant activities (Krinsky, 1993). As different animal taxa may differ radically in their degree of carotenoid uptake (Parker, 1996), the relative importance, and the roles, of carotenoids are, therefore, likely to be very different among species (Perez-Rodríguez, 2009). Some recent

studies have even suggested that carotenoids are insignificant as antioxidants in certain animal taxa (Costantini & Møller, 2008; Perez-Rodriguez et al., 2008).

1.1.6. Carotenoids and immune response

Related to their function as antioxidants is the role carotenoids play in the innate (i.e., cellular or non-specific) branch of the immune response (Chew & Park, 2004). When white blood cells (leukocytes) encounter invading microorganisms, they produce large amounts of peroxides and radicals that subsequently destroy the invading microorganism, processes known as respiratory bursts and phagocytosis, respectively (Eberhardt, 2000; Chew & Park, 2004). The presence of carotenoids protects both white blood cells and surrounding tissues from the harmful effects of these ROMs (Bendich, 1989). Importantly, carotenoids may also stimulate the immune system in ways not connected to their antioxidant activity (for comprehensive reviews of the many immunomodulating effects of carotenoids, see Bendich, 1989; Bendich & Olson, 1989; Chew, 1993; Chew & Park, 2004).

1.1.7. Carotenoids and the developing embryo

The carotenoids present within the eggs of many animals may, at first, seem outside the scope of this review. However, the evolution of mechanisms for incorporating carotenoids into eggs may have been starting points for signal evolution (see section 5.2, The demands of the egg). Furthermore, in species with female ornaments, signal expression may be directly traded off against egg production (Figure 2). Carotenoids are largely responsible for the rich coloration of egg yolk in birds and the eggs of many fishes (Craik, 1985; Blount et al., 2000). For over a century, the unusually high concentration of carotenoids in eggs has been recognized, and their importance and function still constitute a large part of carotenoid research (Palmer & Kempster, 1919; Steven, 1949; Blount et al., 2000). One possible explanation is that the antioxidant function of carotenoids may offer protection from free radical induced cell damage during the sensitive stage of embryonic development (Surai & Speake, 1998; Karadas et al., 2005). In fact, most support for antioxidant activity of carotenoids come from studies of embryos and younger juveniles (Surai, 2002). For example, in grass shrimps (*Palaemonetes pugio*), egg carotenoids (astaxanthin and β -carotene) decrease during embryo development, whereas other antioxidants, such as enzymes, increase (Winston et

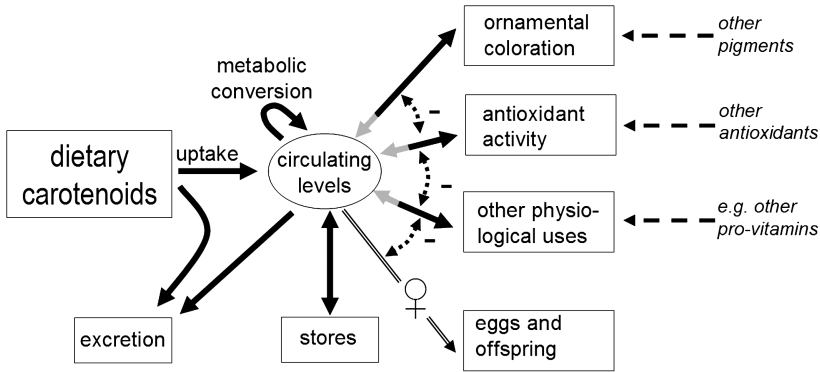


Figure 2. A flow chart of carotenoid functions in ornamented animals highlighting the many and complex interactions that can be expected. In most animals, precious little is known in detail about these processes. After uptake and possible metabolic conversion, carotenoids are circulated in the blood. Carotenoids may then be stored or allocated to different functions. If used in ornamentation, they may become irretrievably deposited in tissues, such as in bird plumage. They may also be used as antioxidants, either as catalysts or irreversibly depleted. All these processes may favour certain carotenoids due to carotenoid-specific enzymes and lipoproteins. Grey arrowheads indicate partial bi-directionality. Dotted arrows are examples of internal trade-offs. Hollow arrows indicate female-only processes. The dashed arrows illustrate how carotenoids share most of their functions with other compounds. In fact, few, if any, functions are uniquely attributable to carotenoids. For an example of the full complexity of the carotenoid dynamics within a species, see Rajasingh et al. (2006).

al., 2004). Thus, it is likely that maternally-acquired carotenoids have the greatest role early on in development, before more complex antioxidant systems are assembled. The polyunsaturated lipids found in the eggs of many organisms are prone to oxidation and can rapidly be broken down by oxidants (Østerlie, 2000). Such oxidants are, in turn, a natural consequence of the fast metabolism of developing embryos, and carotenoids can, therefore, offer important protection from oxidative stress (Miki, 1991; Okimasu et al., 1992). The combination of high growth rates and sensitive tissues may be an evolutionary explanation for the high carotenoid concentrations seen in eggs (Blount et al., 2000). The low oxygen tension typical in avian embryos is also thought to favour the antioxidant activity of carotenoids (Krinsky, 1993; Surai & Speake, 1998). It is important to note that egg carotenoid concentrations vary greatly between species (Blount, 2004). Interestingly, some animals have eggs that are virtually devoid of carotenoids (Rønnestad et al., 1998), and this may be true despite a carotenoid rich diet (Svensson et al., 2009).

1.1.8. Additional physiological effects of carotenoids

Carotenoids are important as precursors to vitamin A in many animals (Moore, 1930; Morton & Creed, 1939; Liñán-Cabello et al., 2002). This function is accredited to about 10% of carotenoids (Krinsky, 1989). A carotenoid molecule split in half will, somewhat simplified, produce two vitamin A molecules (Parker, 1996). Vitamin A, in turn, is vital for many key functions, including cell proliferation during development (Maden, 1993) and production of visual pigments (Bowmaker, 1995). It is important to realize, however, that animals differ greatly in their ability to convert carotenoids into vitamin A (Parker, 1996), and there are taxonomic differences in which forms of vitamin A that are used (Lubzens et al., 2003).

Apart from acting as antioxidants and provitamin A, carotenoids have many other important physiological functions. They have a role in inhibiting mutagenesis (Bendich & Olson, 1989), in regulating membrane fluidity (Chew & Park, 2004) and in cell communication by regulating the synthesis of connexin 43, a protein used in intercellular gap-junctions (Zhang et al., 1992). Xanthophylls, in particular, are capable of physically stabilizing the phospholipid cell membrane and, therefore, protect cell integrity (Young & Lowe, 2001; Britton, 2008). Carotenoids can also be important in photo-protection, that is, shielding sensitive tissues from the oxidising effects of sunlight, in particular, UV radiation (Byron, 1982; Britton, 2008).

Dietary carotenoid supplementation has demonstrated several positive effects *in vivo* where the exact mechanism is either unknown or where the causality is uncertain (called carotenoid actions and carotenoid associations, respectively, Krinsky, 1993). For example, carotenoid supplementation has been shown to improve life-history traits like condition (Smith et al., 2007), growth (Torrissen & Christiansen, 1995; George et al., 2001; Biard et al., 2006), survival (George et al., 2001; Saino et al., 2003; Chien & Shiau, 2005; Pike et al., 2007a) and parental care (Pike et al., 2007c) in a wide range of taxa.

It is important to mention that, apart from their roles in photosynthesis, carotenoids do not appear to have any functions uniquely attributable to them. For example, similar pigmentation (at least with respect to the human visual system) may be achieved with other compounds, such as melanins, flavonoids, haemoglobin, porphyrins, psittacofulvins, pterins and even structural colours (see, e.g., Toral et al., 2008). Carotenoids also share their role as animal antioxidants with many vitamins and antioxidant enzymes (Figure 2).

1.2. *Signals and signal honesty*

A major focus of carotenoids in behavioural ecology has been on their role in signalling. In this section, we will provide a general introduction to signalling theory relevant to the subsequent discussion.

1.2.1. *What are signals?*

Most things that organisms want to know about each other cannot be directly observed. For example, an animal may want to assess whether a prey is toxic, if an opponent is physically strong, or if a potential partner is in good health. Under all of these circumstances, assessment requires detecting and interpreting signals and/or cues, which are observable indicators of non-observable qualities.

A biological signal can be defined as “any act or structure which alters the behaviour of other organisms, which evolved because of that effect, and which is effective because the receiver’s response has also evolved” (Maynard Smith & Harper, 2003, p. 15). This is distinct from a cue which, although potentially important in conveying information, has not evolved in order to convey that information (Hasson, 1990). Grey hair is, therefore, not a signal of old age, but it may be used as a cue of old age. Bird song, on the other hand, has evolved specifically to convey information, and is, therefore, defined as a signal. The difference is not always clear cut, however, because the act of displaying a cue can be considered as a signal (Maynard Smith & Harper, 1995; Hasson, 2000).

Many animals have an obvious motivation to deceive each other, and in such cases some mechanism must be in place to ensure the maintenance of signal honesty (Maynard Smith & Harper, 2003). If not, the benefits of deception will cause dishonesty to spread, resulting in communication breakdown. For example, females often use male ornamental traits, such as gaudy coloration, as signals of individual quality in mate choice. While it may be in the females’ interest to accurately estimate male quality, it is in the males’ interest to convince females to mate with them. How can signals remain honest in the presence of such (potentially) conflicting interests?

1.2.2. *Mechanisms maintaining signal honesty*

One suggested mechanism for ensuring honesty is the ‘handicap principle’ (Zahavi, 1975), which states that honest signals must be costly, and that only

high-quality individuals should be able to bear the high cost of extravagant ornaments. Often, it is assumed that the cost is paid via the operation of natural selection (e.g., reduced survival), while the benefit is accrued through sexual selection (e.g., increased access to mating partners). Because the term 'handicap' is ambiguous and potentially misleading (Getty, 2006; Harper, 2006), we will refer to such signals as Zahavian signals, in recognition of the originator. All signals are expected to have some costs associated with transmitting information efficiently to the receiver ('efficacy costs' Guilford & Dawkins, 1991; Maynard Smith & Harper, 2003). Central to the Zahavian signalling idea is that ornaments carry extra, strategic, costs in addition to the efficacy costs. Strategic costs may be incurred when producing the signal structure, such as using energy and nutrients to grow large antlers, or when displaying the signal, such as suffering increased predation risk due to conspicuous coloration.

Integral to Zahavi's theory is that the costs of an ornament create trade-offs within the individual, for example between ornament intensity and health (Zahavi, 1975; Folstad & Karter, 1992). Put differently, animals will experience internal trade-offs because limited resources must be shared between competing demands. An important challenge to researchers is to demonstrate what the costs are, and to understand the nature of the trade-offs. Zahavi's original verbal argument was formalised mathematically by Grafen (1990a,b), leading to an increasing acceptance of the idea (Harper, 2006). Getty (1998a,b, 2002, 2006) has criticised and refined Grafen's models, stressing that signalling contributes to overall fitness in a multiplicative, rather than additive, fashion. Getty's main message has relevance for our understanding of carotenoid-based signals, namely that ornaments are not structures selected to be wasteful. Rather, the physiological adaptations for signalling are expected to be selected for efficiency, just like other investments aimed at future fitness gains (Getty, 2006). Viewed in this way, investing in a signal should be no different to, for example, investing time and energy into establishing a territory, or caching food for later consumption. In this sense, signalling does not have to cause conflict between sexual and natural selection: rather the former should be seen as a subset of the latter. Honest signals of quality could then be maintained because only in high quality individuals will the combined fitness benefits of an intense signal outweigh the costs of the signal itself.

Although the notion of Zahavian signals is popular among biologists, there are important alternative explanations as to how honest signals can evolve and persist (Maynard Smith & Harper, 2003). First, there are cases where there is no conflict between signaller and receiver and, thus, no motivation for deception. Whenever they share a common interest, both will benefit from an accurate signal. A classic example of this is the dance of the honey bee (*Apis mellifera*), which honestly relays information about the location of food to other members of the hive, and where both signaller and receiver benefit from a more accurate signal (von Frisch, 1967). Another example is when certain animals signal their readiness to mate (Rowland et al., 1991; Watkins, 1997; Hager, 2001; Weiss, 2002; Kolm, 2004). In such cases, the benefits of deception may be absent, because mate-searching animals seek to concentrate their courtship effort on receptive individuals, while unreceptive signallers can avoid unwanted sexual attention. Second, honesty can be ensured by the very nature of the signal itself (defined as an index by Maynard Smith & Harper, 1995). An example of this is when funnel web spiders (*Agelenopsis aperta*) vibrate their webs to ward off competitors (Riechert, 1978). The intensity of the vibrations is directly related to body weight, so spiders have no possibility of dishonestly signalling their body size (Maynard Smith & Harper, 1995). Third, dishonesty may exist, but may be uncommon, or may occur only during limited periods of an animal's life (Candolin, 2000a). A fourth, often overlooked, possibility is that dishonesty exists because the signalling system is rapidly evolving, and has not yet reached a signalling equilibrium (Maynard Smith & Harper, 2003).

2. Current standing and controversy

2.1. Carotenoids as signals

In this section we will outline the different contexts in which carotenoid-based signals are used, and summarize the ongoing discussion of the signal content of carotenoid colour patterns, and how their honesty may be maintained. It is important to point out that all carotenoid-based patterns do not have to be signals. Instead, they may result from other processes, such as a passive accumulation of dietary carotenoids, or in order to provide camouflage or protection from sunlight (Byron, 1982; Blount & McGraw, 2008).

2.1.1. Types of carotenoid-based signals

The ability for carotenoids to pigment animal tissue has been known for a long time (e.g., Palmer & Kempster, 1919; Sumner & Fox, 1933). Because carotenoid-based colour patterns are very common, they are now among the most studied of all signals (McGraw, 2006b). They are widespread in the animal kingdom, especially in arthropods, and in vertebrate groups such as fish, reptiles and birds (Blount & McGraw, 2008). Mammals, however, do not express carotenoid-based signals. Indeed, with the exception of ruminants and a few other groups (notably including humans), most mammals do not even absorb carotenoids to any significant extent (Handelman, 2001).

Carotenoid-based pigmentation is used by animals in a variety of contexts, from aggressive displays and species recognition to warning colouration and camouflage (reviewed in Blount & McGraw, 2008). Recently, there has been growing interest in understanding the role of carotenoid pigmentation in parent–offspring communication. Here, both parents and young may express carotenoid-based coloration, examples of which include the red bill spot of adult herring gulls (*Larus argentatus argentatus*) (Tinbergen & Perdeck, 1950) and chick gape coloration of barn swallows (*Hirundo rustica*) (Saino et al., 2000) and hihi (*Notiomystis cincta*) (Thorogood et al., 2008).

Despite the many contexts in which carotenoid-based signals are displayed, none has received as much interest, or is as widely recognised, as those involved in sexual selection. In this regard, carotenoid-based signals — like other kinds of sexually selected traits — can contribute both to intrasexual competition and to mate attraction (Berglund et al., 1996), although this is not always the case. In red-collared widowbirds (*Euplectes ardens*), for example, carotenoid-based signals are used only in male–male conflict and are ignored by females (Pryke et al., 2001). Nevertheless, female preference for carotenoid-based ornaments appears widespread and has been reported in several species of fishes (Kodric-Brown, 1989; Milinski & Bakker, 1990; Houde, 1997; Amundsen & Forsgren, 2001), birds (reviewed in Hill, 1999b; Blount & McGraw, 2008) and at least one reptile (Kwiatkowski & Sullivan, 2002).

2.1.2. Proposed costs and benefits for senders and receivers

To understand *any* signalling system, it is important to address the costs and benefits for both senders and receivers. In several species, a strong preference

for partners with more intense carotenoid pigmentation has been demonstrated, typically in regard to female preferences for more ornamented males (Endler, 1983; Hill, 1991, 1999b). Therefore, an obvious benefit to the sender can be greater access to mating partners. The receiver may also benefit, because the signal may convey important information about the quality of the signaller. The carotenoids allocated to an ornament are typically not believed to benefit the receiver per se. However, such a direct benefit exists in two-spotted gobies (*Gobiusculus flavescens*), where gravid females display their carotenoid pigmented gonads to the male, and males that spawn with more colourful females attain higher-carotenoid eggs for his brood (Svensson et al., 2006).

The costs of carotenoid-based ornaments are thought to affect mainly the sender. Interestingly, over the years there has been a shift in research focus on what types of costs that are incurred (Figure 3). Early studies were often preoccupied with the costly consequences of expressing the signal (e.g., how conspicuous signals relate to predation risk), and how individual differences in the ability to sequester carotenoids from the environment reflect individual quality (Endler, 1980, 1983; Hill, 1991). Later studies, by contrast, have

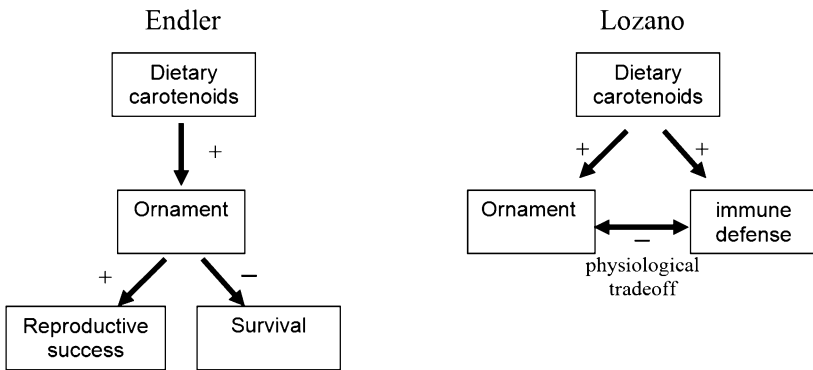


Figure 3. Two approaches to the costs of producing carotenoid-based ornaments. Early studies (left flowchart) typically contrasted the benefits from mate attraction against the costs of conspicuousness, as suggested by Endler (1978, 1980). Following Lozano's trade-off hypothesis (Lozano, 1994, 2001), the main focus has instead been on the physiological costs of producing the ornament in the first place (right flowchart). Importantly, these two approaches are not mutually exclusive. Although these views have been treated as in opposition, the best approach is likely to combine the two (Lozano, 2001). Note that all these processes, including the trade-offs, are predicted to occur within, not across, individuals (Kotiaho, 2001; Getty, 2002).

mainly focussed on the internal (physiological) costs of producing the signal (e.g., trade-offs between signalling and health, Lozano, 1994). This newer focus aims to elucidate how carotenoids, once sequestered, are partitioned between different functions. Importantly, carotenoid-based signals are likely to be costly in both ways (costly production as well as costly consequences), and the two ideas are, therefore, not mutually exclusive (Figure 3).

2.1.3. Carotenoids — not your everyday handicap

Although carotenoids can be important in the physiology of animals (see Introduction), early behavioural studies often overlooked this important fact. Lozano (1994) was the first to underscore the important physiological functions of carotenoids as a key to understanding carotenoid signalling. Zahavian signals are typically thought to improve reproductive success but impair health and survival (Zahavi, 1975; Grafen, 1990a; but see Getty, 2006). For example, conspicuous and cumbersome ornaments, such as the long train of the peacock (*Pavo cristatus*), are often assumed to be directly harmful to the bearer. On the other hand, circulating the high levels of carotenoids required for intense ornamentation is generally believed to *improve* health and survival. Carotenoid-based ornaments are, therefore, not entirely analogous to other ‘handicaps’. To view carotenoids within a Zahavian framework, we need to acknowledge that carotenoids may both indicate individual quality, while, at the same time, contribute to that quality. In other words, carotenoids may both be a signal *component* (by contributing to ornamental display) whilst also influencing signal *content* (by contributing to better health).

If carotenoids have multiple uses, and if the supply is limited, individuals should face trade-offs between their different uses. Although Lozano did not explicitly mention trade-offs in his 1994 paper, his idea of dual uses in display and physiology has subsequently lead to hypotheses that trade-offs must exist between signal expression and health (von Schantz et al., 1999; Lozano, 2001). Thus, instead of carotenoids being detrimental to the animal (e.g., through increased predation risk) researchers began to view them as “beneficial but insufficient” (Olson & Owens, 1998). Lozano proposed that only high quality individuals should be able to reallocate large amounts of carotenoids to their ornaments without impairing their health (Lozano, 1994). Using Getty’s (2006) language: only high quality individuals should invest heavily in carotenoid ornamentation, because only for them will a

stronger signal give sufficient benefits in overall fitness to offset the cost on health.

The idea that costs of ornamentation can be internal and measured in a currency of oxidative stress quickly started to spread in the 1990s (Lozano, 1994; von Schantz et al., 1999), and it gradually overshadowed the earlier focus on predation risk as the major cost of carotenoid ornamentation. Lozano's suggestion was inspired, at the time, by the plethora of studies showing beneficial effects of carotenoid on human nutrition and health (e.g., Bendich, 1989), early research demonstrating that infections can bleach ornaments (e.g., Houde & Torio, 1992), and the idea put forward by Folstad & Karter (1992) that ornaments, curiously often, seem to impair immunocompetence. The notion of trade-offs between health and carotenoid signalling was, importantly, expanded by von Schantz et al. (1999) to include other types of antioxidants and ornaments. The trade-off idea has two testable assumptions. First, carotenoids should have fitness-enhancing effects in addition to those accrued from signalling. Second, carotenoids should be in short supply, so that animals cannot obtain enough for all their needs.

2.1.4. *Linking carotenoid-based signals to health*

The logic underpinning trade-off costs of carotenoid-based signals has led to some confusion in the literature, especially when interpreting correlational studies (Kotiaho, 2001; Getty, 2002; Hōrak et al., 2004). In particular, mistakes can be made when predicting how the cost of a carotenoid ornament should affect the quality it is proposed to indicate. In comparisons *across* individuals, relationships between health and signalling intensity are actually predicted to be positive, due to condition dependence. In other words, high quality individuals are expected to express intense signals as well as have good health (Kotiaho, 2001). *Within* individuals, however, we should expect negative relationships between health and signalling intensity, because of the suggested physiological trade-offs (von Schantz et al., 1999; Getty, 2002; Figure 3). Therefore, it is inappropriate to use correlational studies among individuals to infer patterns of internal trade-offs within individuals. It has often been unclear, however, whether researchers are trying to test for correlations across or trade-offs within individuals (Fenoglio et al., 2002; Svensson et al., 2006; e.g., Aguilera & Amat, 2007; Isaksson et al., 2007; Pike et al., 2007a; Nordeide et al., 2008). If this distinction is overlooked, it

is very difficult to interpret relationships between ornament expression and estimates of quality, such as, for example, infection status (Getty, 2002).

Lozano's actual prediction was that experimental infections should reduce the expression of carotenoid-based ornaments, and conversely, dietary carotenoid supplementation should reduce infection status (Lozano, 1994). The experimental approach is crucial, because individuals are likely to differ both in parasite susceptibility and in their strategies for carotenoid allocation, making correlational studies hard to interpret (e.g., Møller et al., 2000, see also section 4.1, Experimental considerations). Experimental support for Lozano's prediction comes from, for example, male blackbirds (*Turdus merula*), where carotenoid-based bill coloration was dramatically reduced three weeks after an immune challenge (Faivre et al., 2003). Importantly, a subsequent factorial study using experimental infections showed that increased access to carotenoids (a mix of lutein and zeaxanthin) was able to both restore bill colour and reduce parasite growth (Baeta et al., 2008). These studies not only provided evidence for a trade-off between immune response and signalling, but also that carotenoid-based bill coloration is an honest signal of male health. Further experimental evidence for the dual effects of dietary carotenoids on ornament and health have been provided by studies on barn swallow chicks (Saino et al., 2000) and zebra finches (*Taeniopygia guttata*) (Blount et al., 2003; McGraw & Ardia, 2003). Direct evidence of ornament-health trade-offs come from work carried out on guppies (*Poecilia reticulata*) (Grether et al., 2004a), haplochromine cichlids (*Pundamilia nyererei*) (Dijkstra et al., 2007), Siamese fighting fish (*Betta splendens*) (Clotfelter et al., 2007) and red-legged partridges (*Alectoris rufa*) (Blas et al., 2006). Importantly, support for carotenoid-health trade-offs in birds has come from both branches of immune defence (i.e., both cell-mediated immunity and humoral immune response, Hõrak & Saks, 2003). Similarly, β -carotene and astaxanthin are known to improve both types of immune response in rainbow trout (*Oncorhynchus mykiss*) (Amar et al., 2001), and enhance general resistance to infection in salmon (*Salmo salar*) (Christiansen et al., 1995). Thus, at least among fishes and birds, experimental support exists for the link between carotenoids in signals and the positive effects on animal health, as predicted by Lozano and others (Lozano, 1994, 2001; von Schantz et al., 1999).

2.2. *Protection and sparing*

2.2.1. *Ornaments, carotenoids and antioxidant capacity*

Behavioural ecologists are increasingly using biochemistry and physiology as tools for understanding the proximate mechanism behind carotenoid signalling. This represents a much needed break from the past where underlying mechanisms often were overlooked or ignored (the phenotypic gambit of behavioural ecology, *sensu* Grafen, 1984, p. 63). The new approach has resulted in testable hypotheses regarding which particular mechanisms are behind the observed patterns between carotenoids, signals, diet and health. In particular, there has been growing interest in the antioxidant function of carotenoids. This idea is controversial, however, and in 2004, Hartley and Kennedy claimed that we should not assume a direct link between carotenoids, antioxidant activity and health. The basis of their argument was that carotenoids are too precious as ornamental pigments, and too inefficient (or even harmful) to be used in antioxidant defence. Instead Hartley & Kennedy (2004) asserted that carotenoids are highly vulnerable to ROMs, and that carotenoid-based ornaments are used only to indicate how well other, colourless, antioxidants (such as vitamin E and C) protect the carotenoids from oxidation (bleaching). Carotenoids could, therefore, incur a cost, rather than a gain, for the antioxidant budget of animals. Hartley and Kennedy's idea has become known as 'the protection hypothesis' (Perez et al., 2008). Under this hypothesis, carotenoids facilitate signal honesty, not because of their antioxidant properties, but because of their vulnerability. Thus, the level of non-carotenoid antioxidants is the actual quality being signalled, but because those antioxidants are invisible, carotenoids are used as a 'litmus test' of their abundance. Hartley & Kennedy (2004) did not rule out the possibility that carotenoids may be physiologically beneficial in other ways, for example as provitamin A, in embryonic development or in immune defence. However, their main argument was that no specific link exists between the antioxidant and signalling roles of carotenoids. The antioxidant story was, therefore, regarded as a 'red herring' (Hartley & Kennedy, 2004). The main testable prediction of the protection hypothesis is that carotenoids do not contribute significantly to the antioxidant arsenal of animals.

2.2.2. *Sparing*

To experimentally test the protection hypothesis, one must first consider another possibility, namely, homeostatic sparing. As pointed out earlier, differ-

ent antioxidants can function synergistically whilst maintaining the homeostatic balance between oxidants and antioxidants (see section 1.1.3, Oxidant homeostasis, and see also Catoni et al., 2008; Monaghan et al., 2009; Perez-Rodriguez, 2009). Many studies appear to assume that uptake and allocation of carotenoids are passive, non-adaptive processes, so that an increase in oral dose should have direct and proportional effects on the antioxidant status of the tissues. However, organisms are different from test tubes. If animals actively regulate the oxidant-antioxidant balance, we should not expect the effects of dietary input to be so clear cut (Perez-Rodriguez, 2009). Instead, we would expect animals to up-regulate (or reallocate) non-carotenoid antioxidants if carotenoids are scarce, and down-regulate if carotenoids are plentiful. Carotenoids may be spared from their radical quenching work by increased levels of other antioxidants, and, conversely, other antioxidant may be spared by an increased supply of carotenoids (Krinsky, 1989). If an animal is deprived of carotenoids, the immediate consequence may, therefore, not be a proportional reduction in antioxidant capacity, because non-carotenoid antioxidants may be used to fill the void left by the carotenoids (Finkel & Holbrook, 2000; Schwedhelm et al., 2003; Catoni et al., 2008). Conversely, excess of dietary carotenoids may cause animals to reduce the usage of other antioxidants, thereby masking the contribution of the extra carotenoids on total antioxidant activity (Alonso-Alvarez et al., 2004). The immediate consequence of allocating carotenoids to ornaments, may not, therefore, be manifested as reduced antioxidant capacity or an increased oxidative stress in other tissues. Rather, ornamental investment may have other, more obscure, effects, caused by the costs associated with increased production and/or redistribution of alternate antioxidants (von Schantz et al., 1999).

Although a central property of carotenoids is to modulate the levels of other antioxidants *in vivo* (Krinsky, 1993), this important fact has been repeatedly overlooked. An important difference between animals and test tubes is that animals can actively maintain homeostasis. Palozza (1998) reported that, in 9 of 12 studies, dietary carotenoid supplementation led to reduction in plasma levels of vitamin E, and interpreted this as vitamin E being consumed by the toxic workings of the carotenoids. However, an alternative explanation is that less vitamin E was released from bodily stores in animals where the dietary carotenoids assisted in the antioxidant task, thus making vitamin E less needed (von Schantz et al., 1999).

Based on the terminology chosen by von Schantz et al. (1999, p. 5), we name this proposed relationship between ROMs, carotenoids and other antioxidants the ‘sparing hypothesis’. Under this hypothesis, carotenoids may still be ‘protected’ from oxidation by non-carotenoid antioxidants. However, an important distinction is that, unlike the protection hypothesis, carotenoids are recognized as a part of the antioxidant arsenal available to animals, and that the compounds in this arsenal are, to some extent, interchangeable. Accordingly, the sparing hypothesis predicts that increased access to a carotenoid should increase the antioxidant activity attributable to that carotenoid, while reducing the animals’ reliance on other antioxidants. This possibility has been acknowledged in several recent reviews (Catoni et al., 2008; Monaghan et al., 2009; Perez-Rodriguez, 2009), as well as in experimental papers (Bertrand et al., 2006b; Pike et al., 2007b) and is, we believe, central for understanding the roles of carotenoids in animals with carotenoid-based ornaments. Importantly, the crucial and testable difference between the protection and sparing hypotheses is whether or not carotenoids contribute significantly to net antioxidant activity.

2.2.3. *In the wake of the red herring — tests of the protection hypothesis*

Hartley & Kennedy’s (2004) paper introduced a new perspective regarding carotenoids in behavioural ecology, and several attempts to test its prediction has been made. A meta analysis was recently presented by Costantini & Møller (2008), the results of which were used as apparent support for the protection hypothesis. In particular, the authors concluded that carotenoids account for less than 0.002% of the antioxidant capacity in birds. Caution, however, needs to be exercised when interpreting this result. First, the analysis was based on a limited selection of studies, both in terms of number and taxonomic breadth (Perez-Rodriguez, 2009). Second, there were no statistical considerations for mixing different species, study types (correlational vs. experimental), and various antioxidant assays (R. Rosenthal, pers. commun.). Finally, as pointed out by the authors themselves, their conclusion actually ran counter to, and was difficult to reconcile with, the well established antioxidant function of carotenoids reported in embryos and younger birds (Costantini & Møller, 2008).

As Constantini and Møller’s meta-analysis demonstrates, experimentally disentangling the sparing and protection hypotheses can be challenging.

Bertrand et al. (2006b), for example, attempted to test the protection hypothesis by supplementing the diet of zebra finches with a non-carotenoid antioxidant (melatonin). The authors found that melatonin supplementation increased the carotenoid-based colour of the bird's bill, and argued that bill coloration, therefore, signalled non-carotenoid antioxidant levels, as predicted by the protection hypothesis. However, as conceded by the authors, one cannot rule out the possibility that antioxidant sparing was occurring (i.e., that the extra melatonin helped liberate carotenoids from antioxidant defence to ornamentation). A similar study on a fish, (the three-spined stickleback; Pike et al., 2007b), revealed that dietary supplements of vitamin E and C caused more intense male carotenoid ornamentation. Likewise, it was suggested that this result supported the protection hypothesis, but, once again, the authors acknowledged that their data could not differentiate between sparing and protection. Conversely, Karu et al. (2008), found that feather pigmentation was unaffected by vitamin E supplementation in greenfinches (*Carduelis chloris chloris*) and concluded that this contradicted the protection hypothesis. However, as in the previous two examples, the experimental design cannot actually discriminate between the two hypotheses: the findings could just as easily be taken as evidence against the sparing hypothesis.

2.2.4. Carotenoids: contributing antioxidants or not?

Despite the challenges, several studies have managed to produce compelling evidence against the main prediction of the protection hypothesis (i.e., that carotenoids do not contribute to antioxidant defence in animals with carotenoid-based ornaments). In a study on greenfinches by Hōrak et al. (2007) supplementation with a mix of lutein and zeaxanthin led to higher plasma carotenoid concentration and reduced plasma lipid peroxidation. Furthermore, although the dietary carotenoids did not affect the strength of immune response, they partially alleviated the oxidative stress associated with it. Interestingly, an endogenous antioxidant, uric acid, appeared highly important for the total antioxidant activity in this system, whereas vitamin E was not (Hōrak et al., 2007). This study demonstrated a real antioxidant function of carotenoids, and also highlighted the importance of measuring several antioxidants and using a multi-pronged approach when quantifying antioxidant activity. Another convincing example was provided by a factorial, dose-response study on zebra finches (Alonso-Alvarez et al., 2004). This study showed that immune activation diverted carotenoids from plasma and,

conversely, that increased plasma carotenoids elevated plasma antioxidant activity. Interestingly, the important factor was not the carotenoid (a mix of lutein and zeaxanthin) dose per se, but the resulting change in plasma carotenoids, demonstrating that individual differences in uptake and/or allocation strategies may obscure direct effects of carotenoid supplementation (Alonso-Alvarez et al., 2004). Similar support comes from red-legged partridges, where those males that maintained intense coloration despite testosterone injection suffered increased oxidative damage, whereas males that retrieved carotenoids from the ornament could avoid oxidative stress (Alonso-Alvarez et al., 2008). In female zebra finches, the oxidative stress incurred by producing many eggs was alleviated by dietary carotenoids (a mix of lutein and zeaxanthin, Bertrand et al., 2006a).

In contrast to the aforementioned examples, it is important to point out that some studies have failed to detect antioxidant effects of carotenoid supplementation (e.g., Costantini & Møller, 2008 and references therein). Hōrak and co-workers (2010), for instance, recently found that carotenoid supplements (lutein) given to green finches did not reduce indicators of oxidative damage in blood plasma. It is, therefore, unclear exactly how *general* the antioxidant role of carotenoids might be among animals (see section 3.3, How much can be generalized). Nonetheless, studies in which carotenoids have been shown to contribute to the antioxidant arsenal of species with carotenoid-based ornaments pose a serious challenge to the protection hypothesis.

2.2.5. *Support for sparing?*

Demonstrating important antioxidant effects of carotenoids, however, does not automatically provide evidence in support of the sparing hypothesis. Sparing requires carotenoids to function so similarly to other antioxidants that they can, and will, substitute each other. Because antioxidants have quite different chemistry, the extent of such interchangeability will naturally be contingent on which compounds are being considered (see section 1.1.1, Carotenoid chemistry). However, we believe that antioxidant substitution should not be ruled out as a possibility a priori. Direct experimental evidence for sparing between ornamental carotenoids and other antioxidants comes from a field study of yellow-legged gulls (*Larus michahellis*). Here, vitamin E supplementation led to increased bill ornamentation as well as an elevated plasma carotenoid concentration (Perez et al., 2008). Importantly, only

those particular carotenoids used in the ornament increased in the plasma. This result fits well with the idea that the presence of extra vitamin E spared those carotenoids, so that they could be deposited in the ornament without disrupting oxidant homeostasis.

Indirect evidence of sparing comes from animals that rely more heavily on carotenoids during times of limited access to other antioxidants, as seen, for example, in developing young where antioxidant enzymes are yet to be synthesized (Winston et al., 2004). In blue tits (*Cyanistes caeruleus*), carotenoid supplementation (a mix of lutein and zeaxanthin) did not affect plumage colour or plasma carotenoid concentrations, but it almost halved the circulating levels of vitamin E (p. 1008 and figure 2A in Biard et al., 2006), suggesting that these carotenoids spared other antioxidants. In hihi chicks, increased dietary carotenoids (a mix of lutein and zeaxanthin) improved growth and led to increased plasma levels of carotenoids and reduced plasma levels of vitamin E (Ewen et al., 2006). Although these studies appear to provide support for the sparing hypothesis, the non-adaptive alternative, suggested by Palozza (1998), is also possible: namely that carotenoids are detrimental, thereby leading to increased consumption of vitamin E. However, the ample biochemical evidence for synergy between carotenoids and vitamin E, and the scant support of prooxidant effects of carotenoids *in vivo* (see below), make this non-adaptive explanation unlikely.

If sparing is occurring, the prediction is that although carotenoid supplementation may reduce, for example, vitamin E concentration in certain tissues, the whole-animal reservoir should not decrease. This has been demonstrated in chickens (*Gallus domesticus*), where carotenoid supplements (a mix of lutein, citranaxanthin, canthaxanthin and β -apo-8'-carotenoid acid) increased vitamin E levels in the liver, their main storage tissue (Surai & Speake, 1998). Regrettably, many studies only assay one tissue type, typically plasma, which may make deductions about sparing difficult (see section 4.1, Experimental considerations).

In conclusion, recent studies have shown that carotenoid-based ornaments can, indeed, signal antioxidant capacity, but, importantly, that this capacity includes the effects of the carotenoids themselves. The relative value of carotenoids is likely to vary greatly between species (Catoni et al., 2008; Perez-Rodriguez, 2009). Even within species, studies may yield conflicting results, as in green finches where carotenoids have been found to be both

important (Hörak et al., 2007) and unimportant (Hörak et al., 2010) for antioxidant defence. However, in light of the evidence, we strongly discourage any sweeping statements that carotenoids are ‘generally unimportant as antioxidants’ (especially if referring to large taxonomic groups). The challenge is now to test the generality of proposed mechanisms, and to determine whether the antioxidant function of carotenoids is likely to have actually influenced the evolution of sexual signals. Finally, it is important to remember that it is not all about the antioxidant function: carotenoids may be beneficial to animals in many other ways, as suggested by Hartley & Kennedy (2004) and reviewed in the Introduction.

3. Unresolved issues

While some facets of carotenoid-based ornaments and signalling have received considerable attention during the last three decades, other topics have attracted very little research focus. In the following section, we highlight a few of the questions that have generally been overlooked but are, nonetheless, important in developing our understanding of the field.

3.1. *Does carotenoid access ultimately limit signal expression?*

A central tenet for the trade-off hypothesis is that carotenoids are in limited supply. Support for this come from decade-old observations that captive animals eventually become less pigmented than their wild counterparts, but that this can be rectified by supplementation with appropriate carotenoids (Hill, 2006). Consequently, carotenoid supplements are now common in pet food, as well as in poultry and fish farming (Blount & McGraw, 2008). Although this provides evidence that dietary carotenoid limitation can exist, it does not answer whether animals in the wild are limited by their natural diets. Regrettably, few studies have investigated carotenoid limitation in the wild (Monaghan et al., 2009). It is problematic to use results from captive animals, or extrapolations between species, to infer that dietary carotenoid access is limiting signal expression (Perez-Rodriguez, 2009). This is because dietary access, signal intensity, carotenoid requirements and antioxidant machinery are all known to vary enormously between species, and even between populations of the same species (Endler, 1980; Hill, 1999a, 2006; Surai et al., 2001a; Olson & Owens, 2005; Catoni et al., 2008). However, even if carotenoid limitation can be demonstrated in the wild, a bigger issue still

remains. Namely, although dietary limitation may well be demonstrated on a proximate (physiological) level, this is altogether different from trying to explain limitation of signal expression on an ultimate (evolutionary) level. In other words, exploring what can limit signal expression in individuals is fundamentally different from asking how the signal intensity is maintained (or not) over several generations. Very few studies have explicitly addressed what may ultimately be limiting the exaggeration of carotenoid-based ornaments over evolutionary time.

The implication of this discrepancy is underscored by studies carried out on two morphs of Pacific salmon (*Oncorhynchus nerka*). Both morphs, kokanee and sockeye, attain intense red ornamentation (Craig & Foote, 2001; Foote et al., 2004). Individuals vary in colour intensity, just as we would expect from wild animals with limited access to carotenoids. However, lake-living kokanee has an extremely carotenoid-poor insect diet, while the seafaring sockeye can access much richer foods. If a sockeye is forced to reside in a lake, the carotenoid poor diet will render it completely unornamented. The kokanee, however, is three times more effective than the sockeye at absorbing carotenoids, and become just as red as seafaring sockeye, despite their carotenoid poor lake diet (Craig & Foote, 2001). This dramatic evolutionary enhancement of carotenoid utilization is remarkable, considering that the ancestor (sockeye) already was extremely efficient at using carotenoids compared to other species (Rajasingh et al., 2007). Apparently, strong sexual preferences for colourful partners has fast-tracked the evolution of this new, hyper-effective, carotenoid machinery in a mere few thousand years (Foote et al., 2004). This has also happened more than once, because the kokanee morph has evolved repeatedly and independently in several lakes (Taylor et al., 1996). This example raises a number of important questions: Given their ample access to richer carotenoids sources, and considering the reproductive disadvantage of being drab, why are not all seafaring sockeye maximally colourful? Put differently, if carotenoid metabolism is so highly adaptable (Rajasingh et al., 2007), what is keeping sockeye individuals from also evolving a more efficient usage? Can dietary access to carotenoids really be considered to limit sockeye signal intensity when their closest relatives manage to be equally colourful on a much poorer diet?

Important differences in carotenoid utilization have also been shown in other closely related species. For example, chicks of blue and great tits (*Parus major*) respond very differently to carotenoid supplementation (a mix

of lutein and zeaxanthin, Biard et al., 2006). Among New World orioles, certain species have independently lost otherwise ubiquitous carotenoid coloration in exchange for melanin-based patterns (Hofmann et al., 2007). Artificial selection can also rapidly create substantial differences in carotenoid utilization. For example, domestic chickens use carotenoids to colour their beaks, combs and legs, but their wild ancestor, the red jungle fowl (*Gallus gallus*), does not (McGraw & Klasing, 2006). In various pets, such as ornamental fishes, artificial morphs with and without carotenoid-based pigmentation can rapidly evolve through artificial selection (Clotfelter et al., 2007). Thus, it appears that carotenoid utilization is evolutionary plastic, and may be drastically modified in a fairly short time. Very little evolutionary innovation is needed to dramatically change the way that, and to what degree, carotenoids are utilized (Rajasingh et al., 2007). Possibly as a consequence, the relationship between dietary carotenoid levels and signal intensity is enormously variable between species. Recent studies of the avian family Icteridae have shown that the type of pigment used in ornaments is evolutionary labile, that carotenoid colours have evolved multiple times, and that even closely related clades can take drastically different directions in their signal evolution (Hofmann et al., 2006; Kiere et al., 2009). We encourage future studies to use quantitative genetic methods to investigate the genetic architecture behind carotenoid-based signal traits. It would be particularly interesting to quantify the evolvability and the degree of additive genetic variance of carotenoid pigmentation in different taxa.

Given the evolutionary plasticity, one may assume that exaggeration of carotenoid pigmentation (driven by sexual selection through mate preferences) should go on forever. One obvious reason why this does not occur is that when tissues become saturated with pigment, the concentration-coloration relationship will eventually level off. Ornament intensity will, therefore, become an increasingly inaccurate indicator of tissue concentration, and the force of selection should diminish as the signal loses reliability. We may speculate that certain invariable and highly saturated animal colour patterns could, in fact, have been 'past ornaments' that have since reached fixation in this fashion. However, typical carotenoid-based ornaments are not at this point because they will, generally, demonstrate condition dependent expression (Griffith et al., 2006) and positive responses to dietary supplements (Blount & McGraw, 2008). The question, therefore, remains: could some processes other than dietary limitation ultimately restrict the exaggeration of carotenoid-based ornaments?

3.2. Other costs that may limit carotenoid ornamentation

The strong focus on trade-offs in recent years has overshadowed the fact that carotenoid-based signals may incur other types of costs, and that these may better explain the evolution and maintenance of signal honesty. In particular, there can be various costly consequences associated with expressing carotenoid ornaments, similar to other, more classic, Zahavian signals.

First, it has been suggested that too high concentrations of carotenoids may be directly harmful to animals (e.g., Burton & Ingold, 1984; Britton, 1995). For example, Vinkler & Albrecht (2010) suggested that carotenoids can be harmful to animals due to the toxic cleavage products that may form when carotenoids are broken down. This possibility is supported by studies using mammalian cell cultures, demonstrating toxic effects of carotenoids (or of their breakdown products) at least in very high doses and/or in the absence of other antioxidants (Hurst et al., 2005; Siems et al., 2005; Yeh & Wu, 2006; Kalariya et al., 2008). In vivo, carotenoid supplementation can often lead to a reduction of non-carotenoid antioxidants in plasma, and this has sometimes been interpreted as evidence for carotenoid prooxidant activity (Palozza, 1998). However, without measuring whether those antioxidants are actually consumed or simply diverted, it is impossible to rule out the alternate explanation, namely, that homeostatic sparing is occurring (von Schantz et al., 1999; Monaghan et al., 2009; section 2.2, Protection and sparing). It has also been argued that prooxidant effects of carotenoids are unlikely in living tissue (Young & Lowe, 2001), under physiologically relevant conditions (Krinsky, 1989), or when vitamin C and E are present (Catoni et al., 2008). However, prooxidant effects, or other types of carotenoid-related toxicity, may still be a possibility, considering the extremely high carotenoid concentrations that can occur in certain tissues of some species (e.g., Bjerkgeng et al., 1992; Hatlen et al., 1998; Blount et al., 2002). A recent demonstration of this comes from the extremely colourful American goldfinch (*Carduelis tristis*), where very high oral doses of supplemental carotenoids (a mix of lutein and zeaxanthin) impaired muscle health and flight performance (Huggins et al., 2010). Similarly, in kestrels (*Falco tinnunculus*), high supplemental doses of mainly lutein (which produced extreme concentrations of plasma carotenoids) led to an increased production of ROMs and elevated oxidative stress (Costantini et al., 2007). The ideas of directly harmful effects of carotenoids can, therefore, not be dismissed. Importantly, any evidence of

toxic effects of carotenoids should, ideally, come from the animal in question, and not extrapolated from extreme carotenoid treatments in other study species, or from in vitro experiments.

Second, increased conspicuousness from carotenoid pigmentation may lead to increased predation risk (Götmark & Olsson, 1997, Figure 3). Despite the fact that this was the first suggested cost of carotenoid pigmentation (Endler, 1978, 1980), the effects of coloration on predation risk has been investigated in very few species. Work on guppies has demonstrated that more ornamented males are preferentially targeted and eaten by predatory cichlids (*Aequidens pulcher*) (Godin & McDonough, 2003). Similarly, copepods with more intense pigmentation (mainly astaxanthin) are consistently targeted by fish predators (Byron, 1982), although, in this particular system, the carotenoids are used for photoprotection rather than signalling.

Third, carotenoid-based ornamentation can carry non-trivial social costs (Rohwer, 1975), similar to, for example, melanin-based status signalling (Senar, 2006). Carotenoid-based ornaments may be used as badges of status, causing less competitive males to acquiesce to more dominant rivals, as shown, for instance, in firemouth cichlids (*Cichlasoma meeki*) (Evans & Norris, 1996), red-collared widow birds (Pryke et al., 2002) and rock sparrows (Griggio et al., 2007). Candolin (2000b) showed that low-quality male sticklebacks were physiologically capable of increasing their carotenoid ornament intensity, but that such dishonesty was curbed by the presence of more dominant rivals. Similarly, in Australian painted dragon lizards (*Ctenophorus pictus*), the social cost for males to display carotenoid-based ornamentation is affected by the number of aggressive neighbours (Healey & Olsson, 2009).

Other costs of carotenoid-based ornamentation are also possible. These may include high-risk foraging to obtain carotenoid-rich food or, in the case of colourful predators, reduced foraging efficiency due to conspicuousness to prospective prey. Another possibility is thermal costs, similar to the orange flavonoid-based signal that reduces heat absorption in otherwise black larvae of wood tiger moths (*Parasemia plantaginis*) (Lindstedt et al., 2009). To our knowledge, none of these have been investigated. It is also possible that mate preferences themselves limit ornamentation. This may occur, for example, if overly ornamented partners are rejected (i.e., stabilizing selection), if mate preferences fluctuate over time (Lehtonen et al., 2009), or if assortative mating is occurring (Elmer et al., 2009). Among birds, many species appear to

have lost their carotenoid-based pigmentation (Hofmann et al., 2007), suggesting that sexual selection may not always drive signal evolution in the same direction. In ornamental displays that have several components, selection may act to preserve a certain balance between carotenoids and the other components of the display, each of which may have their own limitations for exaggeration (Grether et al., 2005, see below). Importantly, carotenoid-based ornaments may incur more than one set of costs, and the type of costs that ultimately limit ornament expression may well vary between species. Different types of costs are also likely to be additive rather than mutually exclusive. There is considerable scope for future studies to delve into costs other than those associated with physiological trade-offs and dietary carotenoid limitation.

3.3. *How much can be generalized?*

Behavioural ecologists have now studied a number of species that exhibit carotenoid-based signals (reviewed in Møller et al., 2000; McGraw, 2006b; Blount & McGraw, 2008). If any general pattern emerges from this information, it is that immense variability exists in regard to carotenoid function. As we have already alluded, controversies over the physiological importance of carotenoids are likely to originate, at least in part, from the vast inter-specific differences in carotenoid machinery (Hill, 1999a; Lozano, 2001). These differences are actually hard to gauge, because most in-depth information about the physiological functions of carotenoids comes from studies on a limited number of taxa: mainly humans and a few other mammalian models (Parker, 1996). Moreover, these studies have tended to focus, rather myopically, on β -carotene (and to a lesser extent on lutein and lycopene, Rao & Rao, 2007). Little is known about the function of other carotenoids in established mammalian models, and even less about their roles in non-mammalian taxa. An important consequence of this is that much existing carotenoid knowledge may be of little direct relevance for understanding animals such as birds and fishes, which often have carotenoid levels several orders of magnitude higher than mammals, and which, typically, utilize different carotenoids (Hill, 1999a; Perez-Rodriguez, 2009). Importantly, mammals do not express carotenoid-based ornamentation (Britton, 2008). This is fundamental, because we should expect additional selection pressures (e.g., from sexual selection) to be present in species that possess carotenoid-based

ornaments. These pressures, in turn, are expected to influence the evolution of carotenoid machinery. Examples of highly different carotenoid utilization in closely related species, as in the Pacific salmon example above, suggest that such evolution can occur rapidly, repeatedly and independently (Craig & Foote, 2001; Biard et al., 2006). Dietary access to carotenoids varies enormously among species, and this may have important consequences for the expression and honesty of carotenoid ornaments (Olson & Owens, 2005). The lack of general patterns between distant taxa is evident if comparing, for instance, birds with mammals (Catoni et al., 2008). Hill (1999a) noted that plasma carotenoid concentrations in ornamented birds are very high compared to certain mammals, and argued that this rules out carotenoid limitation in the former. However, this would assume that both animal groups have a comparable physiological reliance on carotenoids, which appears highly unlikely. Given the important variation in carotenoid uptake, conversion and function that exist among the mammalian models (e.g., Parker, 1996), we should expect species from other taxa to be even more different, especially if they have evolved carotenoid-based ornaments. Carotenoids may even have additional, yet undiscovered, functions in species that are not part of the select few used as models in medicine and biochemistry. Thus, there is a real danger of imprudently comparing 'apples with oranges' (Lozano, 2001).

The relative importance of carotenoids as antioxidants is also likely to vary between taxa (Catoni et al., 2008; Perez-Rodriguez, 2009). A good example of this can be seen in the study of Krinsky (1993) who found that carotenoids can be much less effective as antioxidants compared to vitamin E in some species, but much more effective in others. As a consequence, the contribution of carotenoids to overall antioxidant defence varies greatly between species (e.g., Shapiro et al., 1984; Tsushima et al., 1997).

Important differences in carotenoid uptake, usage and signal expression also exist *within* species, for example between populations, sexes, individuals and even between tissue types of the same individual (Parker, 1996; McKinnon et al., 2000; Surai et al., 2001b; Alonso-Alvarez et al., 2004; McGraw, 2006c; McGraw & Klasing, 2006; Catoni et al., 2008). Population differences in carotenoid metabolism are common, both in the wild but also between lab-bred strains, as exemplified by the inconsistent, and sometimes contradictory, results obtained from studies of captive zebra finches (Collins & ten Cate, 1996; Blount et al., 2003; Forstmeier et al., 2007; Rutkowska, 2007). Males and females can differ dramatically in carotenoid dynamics,

especially in species where females invest large amounts of carotenoids in eggs, while males invest in ornamentation (Grether et al., 2004a; McGraw, 2006c; Rajasingh et al., 2006; Ewen et al., 2008). For example, in female fowl, up to 80% of carotenoids can be stored in the ovaries (Nys, 2000), and in zebra finches, males are able to efficiently take up high doses of carotenoids (a mix of lutein and zeaxanthin) at levels where female uptake has already levelled off (Alonso-Alvarez et al., 2004).

Faced with substantial biological variability, one should endeavour to understand the carotenoid machinery of the specific species under study. Consequently, any inter-specific generalizations and extrapolations about carotenoid function should be made with considerable caution. A promising avenue for future research would be to compare how the roles of carotenoids differ in closely related species, for example, in species pairs with and without carotenoid ornamentation.

3.4. *How do carotenoids relate to other signal components?*

Sexual signals are typically made up of several different components (Candolin, 2003). Yet, it is surprising how often researchers will consider only the carotenoid-based component of a signal, whilst neglecting the others. Here, it is important to realize that the receiver of sexual signals is likely to react to the totality of the display, and that quantifying only one component has the potential to yield an incomplete, or inaccurate, understanding of the signalling system (Johnstone, 1996; Dauwe & Eens, 2008). It is also worth bearing in mind that the relationship between different signal components may not be a simple, additive, one. For example, in Australian painted dragon lizards the presence of carotenoid-based bib coloration is positively condition dependent in the more aggressive red-headed males, but negatively condition dependent in the less aggressive yellow-headed males (Healey & Olsson, 2009). Thus, signal components may relay different messages, and the expression of one may, or may not, be related to that of another (Møller & Pomiankowski, 1993; Candolin, 2003). Even seemingly simple signals may convey more than one message. For example, in male house finches (*Carpodacus mexicanus*), a redder breast patch indicates higher fecundity, while a larger area of the same patch indicates higher viability (Badyaev et al., 2001).

One important consideration is that carotenoid-based colour patterns can also contain non-carotenoid pigments. If so, it raises an interesting question:

if individuals are potentially capable of using other, and potentially 'cheaper' pigments of similar colour, how will the signalling system remain honest (Grether et al., 2004b)? Many fishes, for example, are known to incorporate pteridines into skin chromatophores, often in combination with carotenoids (Armstrong et al., 2000). Pteridines are similar to carotenoids in that they may be yellow, orange or red. Unlike carotenoids, however, they can be synthesized *de novo* by animals. The characteristic orange spot of male guppies, for instance, includes both tunaxanthin, a carotenoid obtained through the diet, and drosopterin, an endogenously produced pteridine pigment (Grether et al., 2001). Intriguingly, in one of the few studies that has considered multiple pigment types in a sexual ornament, there was a positive relationship between pteridine and carotenoid concentrations among guppy populations (Grether et al., 2001). That is, the presence of a second pigment type did not seem to reduce signal honesty in this system. The suggested explanation was that guppy females have evolved such an acute preference for a specific hue, that any evolutionary tampering with the pigment blend is prevented (Grether et al., 2005). Similar mixes of endogenous and exogenous pigments exist in reptiles (e.g., Steffen & McGraw, 2007). For example, Australian painted dragon lizards can obtain either yellow, orange or red heads, depending on the ratio between carotenoid and non-carotenoid constituents of the signal (Olsson et al., 2007; Healey & Olsson, 2009). Birds can incorporate phaeomelanins or psittacofulvins into their plumage (McGraw, 2005), and bare parts of their bodies can be coloured red from haemoglobin rather than carotenoids (McGraw & Klasing, 2006). Importantly, although different classes of pigment may be perceived as being similar in colour, their spectra are typically quite distinct (Toral et al., 2008). Therefore, whether pigmentary substitution is likely will depend on the visual system of the animal and its capacity to discriminate between such hues (as shown in the guppy example above). What is worth emphasizing here is that researchers not actively quantifying non-carotenoid pigments are likely to miss their presence and, hence, their potential importance.

The contribution of carotenoids to an animal's overall appearance may also vary over time. Temporal changes in signal intensity is a common phenomenon as seen, for example, through increased coloration during periods of sexual maturity (Baird, 2004). Importantly, such changes do not have to reflect changes in actual tissue concentration of carotenoids. Cephalopods, crustaceans, fishes, amphibians and reptiles all have chromatophores which

give them the physiological ability to rapidly change colour (Kodric-Brown, 1998). In particular, colour intensity can often be modified during courtship (e.g., Sköld et al., 2008) or aggressive displays (Beeching, 1995). This raises concerns if one attempts to relate skin coloration to carotenoid concentration. For example, in haplochromine cichlids, males change their colours in a few days time if exposed to rivals (Dijkstra et al., 2007), but it is uncertain whether such shifts reflect actual changes in carotenoid concentration, or merely the degree of chromatophore aggregation/dispersion.

Many animals display their ornament during potentially strenuous courtship behaviours, clearly adding to overall signalling costs. Few studies, however, have investigated if the intensity of carotenoid-based ornamentation is related to, or can be compensated through, high courtship intensity. Ideally, future studies should attempt to take a more holistic approach to carotenoid-based signal expression. Understanding more about which processes limit the other signal components may also shed light on the role of the carotenoids themselves.

3.5. *Is it all about sex?*

An overwhelming majority of behavioural studies on carotenoid-based coloration has been concerned with sexual signals. Such studies, although important, belie the fact that carotenoids are deployed across a much wider range of contexts. For example, intense carotenoid pigmentation is common amongst many deep sea animals where their presence are unlikely to be associated with sexual signalling (Wicksten, 1989). More generally, carotenoids are also known to be widely used by many animals in an anti-predator context, including mimicry and aposematism (e.g., Bezzerides et al., 2007; Sandre et al., 2007). Yet, very few studies exist on how the intensity of these non-sexual, carotenoid-based colours relate to diet, antioxidant activity, immunity and internal trade-offs. Outside of sexual selection, it seems that carotenoid-based pigmentation has largely been neglected.

Recent studies of offspring-parent signalling in birds underscore the great research possibilities that exist outside the field of sexual selection (Götmark & Ahlström, 1997; Tschirren et al., 2005; Biard et al., 2006; Fitze & Tschirren, 2006; Helfenstein et al., 2008; Loiseau et al., 2008; Thorogood et al., 2008). Such studies are pertinent for a number of reasons. First, nestling coloration can be carotenoid-based (Thorogood et al., 2008). Second, juveniles suffer high levels of ROM due to extreme growth rates (Surai

et al., 1999, see also Introduction). Third, juveniles may rely on carotenoids more so than adults because the endogenous antioxidant machinery is still under development (e.g., Winston et al., 2004). Finally, the health effects of carotenoids have been much more conclusively shown in early life stages (e.g., Fenoglio et al., 2003; Surai, 2002, see also Introduction).

Another topic of considerable research potential centres around the idea that plants, themselves, may use carotenoids as Zahavian signals of pest defence capacity (Hamilton & Brown, 2001; Blount & McGraw, 2008). Taken together, incorporation of non-sexual signals into the carotenoid discourse should be encouraged, because they are likely to help generalize our understanding of signalling trade-offs and the maintenance of signal honesty.

4. Specific challenges

In the following section, we highlight a number of issues and specific caveats which, in our opinion, should be more widely taken into account by researchers in order to build on the advances that have so far been achieved.

4.1. Experimental considerations

4.1.1. Experimental design

It is important to acknowledge the limitations inherent in correlational studies, particularly when investigating carotenoid-based signals. As we have already seen, Lozanoan trade-offs are hypothesized to occur within, not across, individuals (Kotiaho, 2001; Getty, 2002; Figure 3). Therefore, to relate carotenoid pigmentation to quality among individuals will likely produce ambiguous results (e.g., Møller et al., 2000). A more powerful approach is, of course, to carry out experimental manipulations. However, many studies tend to only manipulate one factor at a time and this, too, can lead to inconsistent results (e.g., Faivre et al., 2003; Costantini & Dell’Omo, 2006; McGraw & Ardia, 2007; Alonso-Alvarez et al., 2008; Perez-Rodriguez et al., 2008). For example, a common approach is to measure changes in ornament intensity after challenging the immune system of the test subjects or, alternatively, by manipulating dietary access to carotenoids or other antioxidants. However, as we have seen above, antioxidant sparing, or release of carotenoids from bodily stores, may mask short-term effects of such manipulations. How can future studies avoid these potential problems?

A superior approach that is gaining favour among researchers is to use factorial designs that manipulate, for example, both dietary carotenoids and immune challenge (e.g., Alonso-Alvarez et al., 2004; Berthouly et al., 2007; Baeta et al., 2008). In this way, one creates a stressful situation (e.g., immune challenge), while also providing the animals with the tool (e.g., a carotenoid) to deal with this challenge. If carotenoids are important, the negative effects caused by the immune challenge should be (partially) alleviated by the dietary supplement, resulting in a significant interaction between the two factors (Kotiaho, 2001). Factorial designs, in this regard, are desirable because they address both the existence of the predicted trade off, and whether carotenoids actually limit ornament expression. If the goal is to understand internal trade-offs, it is also appropriate to use within-subject comparisons, that is, conducting repeated measurements on individuals before, during and after treatments (Perez-Rodriguez, 2009).

A separate consideration is the number of factor levels one should use in supplemental studies. Experiments that have used multiple treatment levels typically find non-linear dose–response curves (Alonso-Alvarez et al., 2004; Ahmadi et al., 2006; McGraw, 2006a). Yet, despite this, most studies typically only employ two treatments (e.g., control vs. supplemented). This has important implications for the interpretation of results because the outcome of two-group designs will be highly dependent on the exact choice of treatment levels. Furthermore, the interpretation of two-level studies tends to assume, often implicitly, a linear response between the two points. A multi-level approach, however, can be important in revealing a variety of non-linear response curves (Conolly & Lutz, 2004). For example, a sigmoid relationship is possible if low concentrations fail to induce any effects. Alternatively, if carotenoids become detrimental at high doses, one might expect to see a quadratic (humped-shaped) response. Importantly, the shape of a response curve may vary between species or sexes, as shown in zebra finches (Alonso-Alvarez et al., 2004). In this regard, experimental designs that allow detection of non-linear responses may well assist in resolving some of the current controversies concerning the roles and relative importance of carotenoids among and within taxa.

We believe great care should be taken when designing supplemental diets instead of merely relying on manufactured carotenoid mixes. Because the uptake and function of carotenoids are highly specific (Parker, 1996, see also

Introduction), it is important to have identified the major dietary and ornamental carotenoids of the system. Ideally, this should include information about the ratios and functional differences of different isomeric forms (e.g., Østerlie et al., 1999). The experimental outcome will only be biologically relevant if the selection of supplemental carotenoids has been based on such knowledge. Fortunately, an increasing number of carotenoids (natural and/or synthetic) are becoming commercially available. Knowledge about the animal's natural carotenoid profile is also important when it comes to analysing tissue samples. As we have already pointed out, animals can, and often do, convert one carotenoid into another after uptake, and different carotenoids may spare each other (see section 2.2, Protection and sparing). Therefore, it may be insufficient to only measure tissue concentrations of the supplemented carotenoid(s), or, indeed, to only quantify those carotenoid(s) incorporated into the ornament (see, e.g., Cucco et al., 2007).

4.1.2. *Moving beyond a single-tissue, single-sample approach*

Researchers are often interested in how ornamentation relates to the carotenoid budget of the whole animal. Yet, many studies typically only quantify carotenoids in a single tissue type. This is problematic because the concentration of carotenoids (or other antioxidants) in one type of tissue may often be unrelated to that of another (Catoni et al., 2008). Furthermore, both immune stress and carotenoid supplementation are known to cause redistribution of carotenoids between different types of tissues. For example, Koutsos et al. (2003) found that immune challenged chickens had reduced liver and plasma carotenoids, but increased carotenoids in the thymus and bursa. Thus, in order to fully understand carotenoid usage, a more appropriate approach would be to analyse multiple tissue types simultaneously (Bjerkeng et al., 1999; Surai et al., 2001b).

Problems associated with a single-tissue, single sample approach is exemplified by studies that focus exclusively on measuring carotenoids in plasma. Quantifying carotenoid levels from blood plasma is especially common in avian studies (perhaps with the exception of poultry research), because sacrificing animals may not be permitted or desirable. However, reliance on plasma can be difficult, because it is typically unknown how well, if at all, plasma carotenoids reflect overall levels of carotenoids in the body (Perez-Rodriguez, 2009). Large amounts of carotenoids, for example, can be stored in tissues such as gonads, skin and liver (Surai et al., 2001b; Rajasingh et

al., 2006). Yet, the relative amount of carotenoids present in such tissues is rarely known. It is important to emphasize that plasma carotenoids are, to a large extent, carotenoids in transit. Plasma carotenoid levels are expected to increase when carotenoid access outweighs use, and decrease when use outweighs access (von Schantz et al., 1999). Thus, although plasma carotenoids can tell us about the relative balance between uptake and deposition, they reveal little about the absolute magnitude of either of these processes or, indeed, the overall reservoir available to the animal (see e.g., Mougeot et al., 2007). Possibly because of this, plasma levels can be notoriously inaccurate proxies of carotenoid uptake and deposition, even within the same species (cf. Storebakken & Goswami, 1996; Wathne et al., 1998). Moreover, plasma levels only estimate carotenoids circulating in the extracellular component of the blood, where they are exclusively associated with lipoproteins which can have very high affinities for particular carotenoids (Parker, 1996). Plasma carotenoid concentration is known to be highly variable over different time scales, and is affected by foraging, infections, egg laying and moulting. For example, carotenoids can be rapidly remobilised to plasma following an immune challenge (Costantini & Dell’Omo, 2006). If only a single plasma sample is taken, one risks missing such fluctuations.

When investigating antioxidant activity, plasma assays may also be inadequate, because they measure only extracellular antioxidants (Perez-Rodriguez et al., 2008) and antioxidant enzymes may be lacking or have limited activity (Costantini, 2008). Plasma may, therefore, differ substantially from other tissue types in the balance between classes of antioxidants. Symptomatically, carotenoid supplements can lead to increased plasma levels of some antioxidants but reduced levels of others (Ewen et al., 2006; Morales et al., 2008).

A multiple-tissue approach should, therefore, be preferable to a plasma-only approach in most situations. If dissection (or biopsies, Zebisch et al., 2004), cannot be performed to sample different tissues, serial plasma samples may be a minimum requirement for accurately capturing changes in carotenoid allocation, and teasing apart alternative explanations (Perez-Rodriguez, 2009). It may also be informative to estimate ‘residual plasma carotenoids’, that is, the concentration of circulating carotenoids not explained by ornament intensity (Blas et al., 2006). Ideally, it would be best to increase the use of model species where it is feasible to assay all the major storage tissues. Future studies that attempt a whole-animal approach will likely help resolve many of the current controversies.

4.1.3. *Manipulating allocation rather than supply*

Most early attempts at testing Lozano's idea of physiological trade-offs tended to rely on simple experimental supplementation of the diet. In this regard, a common treatment would be to increase carotenoids in food or drinking water, usually with increased coloration as a result. However, this approach (and the ensuing results) does not really provide a test of Lozano's predictions (see section 2.1.4, Linking carotenoid-based signals to health). Rather, such experiments only go as far as addressing Endler's older idea that ornament intensity should reflect dietary access to carotenoids (Figure 3). To test for an actual Lozanoan trade-off, one should try to manipulate the allocation of existing dietary carotenoids to its different uses, instead of the overall supply. This, regrettably, is a much more challenging task. Nevertheless, as recent studies have demonstrated, a number of viable methods can potentially be used for manipulating carotenoid allocation to ornaments, including the use of artificial morphs differing in carotenoid pigmentation (Clotfelter et al., 2007), manipulation of the social environment (Candolin, 2000b; Dijkstra et al., 2007) or administration of testosterone (Jayasooriya et al., 2002; Peters, 2007).

Given the challenges of directly manipulating investment into ornaments, it may be easier to instead manipulate the allocation of carotenoids to other uses than ornamentation. For example, if an animal's immune system is challenged, this is expected to divert carotenoids away from other uses such as ornamentation. A more powerful approach is, once again, to use factorial designs to manipulate both access and allocation concomitantly (see section 4.1.1, Experimental design).

4.1.4. *Important first steps when investigating carotenoid-based signals*

As we have already highlighted, it would be beneficial if all investigations built on a solid biological understanding of their particular study systems. As a starting point, it is prudent to determine whether, in fact, the signal of interest is actually carotenoid-based and, if so, which carotenoids, and which other pigment types, are involved in the ornament. It would also be sensible to determine which carotenoids are present in the natural diet of the study organism, which of these are actually taken up in the animal, in which tissues they are being deposited, and whether any metabolic conversions of carotenoids are taking place. For example, even if carotenoid supplementation increases ornament intensity, this is not conclusive evidence

that the used carotenoid is actually present in the ornament. If the dietary carotenoid has an antioxidant function, it may instead have spared the actual ornamental carotenoids. More broadly, from a natural history perspective, researchers may need to also ask whether a species is likely to be carotenoid limited in the first place, and whether there are sex, age or seasonal differences in carotenoid access, uptake, need and usage. Finally, in the context of designing good experiments, it may be necessary to establish dose-response curves for uptake and ornamentation using biologically relevant carotenoids at appropriate levels.

4.2. *Appreciating the complexity of homeostatic systems*

4.2.1. *The antioxidant system*

Many studies aim to understand the links between carotenoid-based ornaments, oxidative stress and the antioxidant capacity of an animal. Several different techniques exist for measuring antioxidant capacity, each with their own limitations (Perez-Rodriguez, 2009). Similarly, several assays exist for quantifying oxidative stress (reviewed in Monaghan et al., 2009), and these may produce conflicting results (Costantini, 2008; Costantini et al., 2008). Thus, for any chosen method, it is necessary to understand its limitations and the biochemistry and physiology behind it. Without such knowledge, it may be inherently difficult to interpret results and infer causality in well-balanced, homeostatic systems. One potentially powerful approach is to simultaneously measure indices for both antioxidant capacity and level of oxidative stress (Costantini & Dell’Omo, 2006) and/or to quantify the amount of oxidative damage, for example by measuring the amount of peroxidation products (Hörak et al., 2007). Multi-pronged approaches may reveal vital information about whether changes in antioxidant levels are likely to compensate partially, completely, or even hormetically (i.e., over-compensating, Conolly & Lutz, 2004) when individuals are responding to oxidative stress. Researchers specifically interested in testing Lozano’s trade-off hypothesis should ideally quantify the antioxidant activity directly attributable to the ornamental carotenoid(s), and then relate this to the total antioxidant capacity of the individual (for a review on quantifying oxidative defences, see Perez-Rodriguez, 2009).

4.2.2. *Immune response*

When studying the links between health and carotenoid ornamentation, one quickly realizes that immune function is “not a distinct and easily quantifiable entity” (Hörak et al., 2007, p. 633). Correct interpretations of outcomes from the various available assays may actually require immunological expertise beyond that of most behaviourists. Not only is immune response inherently complicated in itself, but the immune system is partially interlinked with the antioxidant system (Perez-Rodriguez, 2009). In this regard, it can be hard to distinguish an adaptive response from a non-adaptive (detrimental) side effect. For example, ROM production is an effective way to kill pathogens, but it can also damage surrounding tissues, potentially making even mild inflammatory responses cause significant oxidative stress (Costantini & Dell’Omo, 2006). Because phagocytosis relies on oxidants (ROM), excess plasma carotenoids may, in fact, impair the immune system by counteracting the ROM (Krinsky, 1974; Zhao et al., 1998). This was precisely what McGraw & Klasing (2006) found in a study of red junglefowl given a mix of lutein and zeaxanthin (although, in that study, the authors speculated that the inhibition of the phagocytosis by these carotenoids was due to carotenoid toxicity rather than carotenoids neutralizing ROM). It is important to realise that immune challenge can lead to a concurrent increase in both oxidative damage and total antioxidant capacity (Hörak et al., 2007), which is expected from an incomplete mitigation of the negative side-effects of phagocytosis.

Different species have very different immune responses, and there are also trade-offs between different branches of the immune system (Hörak et al., 2006; Matson et al., 2006). Quantifying any single proxy of immune function may, therefore, yield an incomplete picture. Ideally, studies should quantify both humoral and cell-mediated immune responses (Hörak & Saks, 2003). When this has actually been carried out, important discrepancies have been found between the different types of immune response in how they react to changes in carotenoid levels (McGraw et al., 2006b; Aguilera & Amat, 2007). Consequently, estimates of the immunoregulatory role of carotenoids may be highly sensitive to which immunoassay is actually employed and to which branch of the immune system is being measured (Saino et al., 2003). Not surprisingly, there has recently been a call for using standardized immune tests when measuring responses to immune challenge (McGraw et al., 2006b), and also for using multiple immune tests (Hörak et al., 2006).

5. Some future perspectives

In this section we outline two specific themes we believe should be pursued in future studies, as they may explain both proximate and ultimate reasons for the presence of carotenoid pigmentation.

5.1. *Testosterone: a missing link?*

Recent empirical studies confirm that testosterone, the principal male sex hormone in vertebrates, might be an important missing link between carotenoid-based ornaments and immune defence, at least in male birds (reviewed in Peters, 2007) and certain fishes (Bjerkeng et al., 1999; Kurtz et al., 2007). It has long been suggested that steroid hormones affect oxidative stress, thereby mediating signal honesty (Folstad & Karter, 1992; von Schantz et al., 1999). Testosterone may also be important in birds where females express a less intense version of an ornament expressed by males. For example, in male zebra finches, the negative effects of testosterone are ameliorated by a simultaneous increase in carotenoid uptake, but this does not occur in females (McGraw, 2006c). One possible mechanism for this may be that testosterone elevates plasma carotenoids by upregulating lipoprotein production (McGraw et al., 2006a; but see Casagrande et al., 2010).

In males, high testosterone levels may be a necessary nuisance for achieving impressive ornamental structures, such as big tails (Folstad & Karter, 1992), and/or sufficient levels of aggression. However, increased testosterone can induce increased carotenoid uptake (McGraw et al., 2006a), either as a mere side effect, or possibly as an adaptive defence against the negative effects of testosterone (Blas et al., 2006). Building on recent results (Blas et al., 2006; McGraw et al., 2006a), we suggest that the evolution of carotenoid ornaments (at least in male birds) could have come about through a series of steps. First, males are sexually selected for increasing testosterone levels as this allows them to become more aggressive and/or produce (non-carotenoid) ornaments. This, in turn, selects for improved uptake of carotenoids to combat the oxidative stress caused by the testosterone. Finally, excess carotenoids starts to be deposited in tissues, and become a component of the ornamental display, where the pigment intensity help indicate that a male is both virile and in good health.

A thorough understanding of the roles of testosterone in signal expression would be a significant milestone because testosterone could potentially

serve as a tool to experimentally manipulate the allocation of carotenoids to ornaments (see section 4.1, Experimental considerations). However, it is important to remember that testosterone can have varied and pleiotropic effects on animals, so careful experimentation (e.g., using factorial designs) will be needed to actually ensure that carotenoid uptake and allocation, and not some other response, is responsible for the results. The considerable problem of disentangling cause versus effect and adaptive versus non-adaptive responses in well-balanced systems is highlighted in a recent paper by Vinkler & Albrecht (2010). In addition, both species and individuals are likely to vary in how well carotenoids can mitigate the negative effects of testosterone, possibly explaining the equivocal results when studies have attempted to explore the links between testosterone, signal expression and health (McGraw & Ardia, 2007). The generality of testosterone as an ornament regulator clearly deserves further study, especially in non-avian taxa and/or in the context of female ornaments.

5.2. *The demands of the egg*

The role of carotenoids in embryonic development of many egg-laying species allows us to speculate about whether carotenoid-based ornaments may, in some instances, be by-products from mechanisms evolved for use in egg production. The physiological demands of the developing embryo (Surai & Speake, 1998; Karadas et al., 2005, see also Introduction) may have selected for increased carotenoid deposition into eggs. If a species evolves more efficient mechanisms to absorb, metabolise and use carotenoids, so that females can incorporate them into eggs, males of the same species may find themselves with a carotenoid surplus available for other purposes, such as ornamentation (Rajasingh et al., 2006). Possible support for this hypothesis comes from evidence that taxa with carotenoid-based ornamentation have unusually high carotenoid levels (Hill, 1999a; Olson & Owens, 2005), while, at the same time, carotenoid access is known to limit egg-laying capacity (Blount, 2004). A link between carotenoid use in eggs and ornamentation could help explain the prevalence of carotenoid-based signals in birds, reptiles and fish, as well as their absence in mammals. We believe this idea warrants further study.

6. Summary and conclusions

Carotenoid-based colorations is used by animals in a variety of contexts, from mate attraction and agonistic displays to warning colouration and camouflage. Not surprisingly, carotenoid-based signals have attracted considerable research interest. Carotenoids are also known to perform a raft of physiological functions, particularly in regard to antioxidant defence and immune response. As a likely consequence, the past three decades have witnessed significant shifts in the way researchers view the importance and meaning of carotenoid-based signals. In our opinion, the key to reconciling apparent conflicts (and to avoid some of the pitfalls of past research) is to better address the mechanisms underlying carotenoid-based signals. The different roles performed by carotenoids, and their proposed dietary limitation, are predicted to give rise to internal trade-offs. For example, animals are expected to balance the requirements of carotenoids in ornamentation against their use as antioxidants and immunostimulants. However, when considering immune function and oxidant balance, it is critical to remind ourselves that we are dealing with homeostatically-regulated systems in which carotenoids are but one component. This means animals often will respond in ways that can obscure the effects of experimental manipulations. If one aims to understand how carotenoid-based signals are related to vital components of animal physiology, the experimental designs needs to take this complication into consideration.

Recently it has been proposed that ornamental carotenoids are unimportant as antioxidants and merely indicate the level of other antioxidants in the animal (i.e., the protection hypothesis). Apparent support for this comes from studies that show reduced tissue levels of non-carotenoid antioxidants following carotenoid supplementation. However, if homeostatic regulation is taken into account, another explanation may be possible, namely that non-carotenoid antioxidants have simply been down-regulated in supplemented animals (i.e., the sparing hypothesis). If so, the results of these studies do not contradict studies showing carotenoids acting as important antioxidants.

Substantial variation in carotenoid access, uptake and usage exist, both between and within species. Importantly, in taxa with carotenoid-based ornaments, we should expect additional selection pressures (e.g., from sexual selection), which may drive rapid evolutionary divergence in carotenoid metabolism. At present, the precise mechanisms underlying carotenoid functions are known for only a handful of carotenoids in only a handful of

species. As long as this is the case, great circumspection is required when making generalisations or, indeed, when extrapolating information from one study to another. In particular, experimental studies need to carefully consider the natural history framework of the species, population, sex and/or life stage in question.

Although physiological trade-offs are potentially good at explaining what limits the expression of carotenoid-based signals at the proximate level, such trade-offs do not shed any light on what ultimately limits signal expression. Recent evidence suggests that animals have the capacity to rapidly evolve novel and highly efficient ways to sequester and use carotenoids. This implies that over longer, evolutionary timescales, costs other than dietary access should constrain signal expression. We encourage future studies to pay more attention to other types of costs associated with carotenoid-based signals, such as increased risk of predation or social interactions. The time is also ripe for quantitative genetic studies aimed at describing the genetic architecture and evolutionary constraints of carotenoid-based traits.

When critically examining the carotenoid signal literature, several caveats and pitfalls become apparent. In this review, we have highlighted a number of these with the intent of informing future research efforts. For instance, studies should ideally adopt a whole-animal approach rather than using snapshot measurements of single tissues. More broadly, although we disagree with there being 'too much' focus on carotenoids in behavioural ecology, we do think that there has been far 'too much of the same'. Even at a cursory glance, it is quite apparent that the focus of research has often been biased and lopsided. Taxonomically, the literature is heavily dominated by studies of bird and fish. At the same time, few studies venture beyond the realm of sexual selection. A concerted effort to widen the breadth of studies will no doubt enhance our understanding of the purpose, limits and mechanisms underlying carotenoid-based signals.

Acknowledgements

We thank R. Rosenthal for advice on meta analyses, G. Lozano for a discussion on band-wagons, H. Pärn for talks about trade-offs and U. Candolin and N. Metcalfe for raising the issue of whether behaviourists focus too much on carotenoids. We are grateful to S. Pryke, D. Dowling and two anonymous referees for comments on the manuscript. Funding was provided by the Australian Research Council.

References

- Aguilera, E. & Amat, J.A. (2007). Carotenoids, immune response and the expression of sexual ornaments in male greenfinches (*Carduelis chloris*). — Naturwissenschaften 94: 895-902.
- Ahmadi, M.R., Bazyar, A.A., Safi, S., Ytrestøyl, T. & Bjerkeng, B. (2006). Effects of dietary astaxanthin supplementation on reproductive characteristics of rainbow trout (*Oncorhynchus mykiss*). — J. Appl. Ichthyol. 22: 388-394.
- Alonso-Alvarez, C., Bertrand, S., Devevey, G., Gaillard, M., Prost, J., Faivre, B. & Sorci, G. (2004). An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. — Am. Nat. 164: 651-659.
- Alonso-Alvarez, C., Perez-Rodriguez, L., Mateo, R., Chastel, O. & Vinuela, J. (2008). The oxidation handicap hypothesis and the carotenoid allocation trade-off. — J. Evol. Biol. 21: 1789-1797.
- Amar, E.C., Kiron, V., Satoh, S. & Watanabe, T. (2001). Influence of various dietary synthetic carotenoids on bio-defence mechanisms in rainbow trout, *Oncorhynchus mykiss* (Walbaum). — Aquacult. Res. 32: 162-173.
- Amundsen, T. & Forsgren, E. (2001). Male mate choice selects for female coloration in a fish. — Proc. Natl. Acad. Sci. USA 98: 13155-13160.
- Armstrong, T.N., Cronin, T.W. & Bradley, B.P. (2000). Microspectrophotometric analysis of intact chromatophores of the Japanese medaka, *Oryzias latipes*. — Pigment Cell Res. 13: 116-119.
- Badyaev, A.V., Hill, G.E., Dunn, P.O. & Glen, J.C. (2001). Plumage color as a composite trait: developmental and functional integration of sexual ornamentation. — Am. Nat. 158: 221-235.
- Baeta, R., Faivre, B., Motreuil, S., Gaillard, M. & Moreau, J. (2008). Carotenoid trade-off between parasitic resistance and sexual display: an experimental study in the blackbird (*Turdus merula*). — Proc. Roy. Soc. Lond. B: Biol. 275: 427-434.
- Baird, T.A. (2004). Reproductive coloration in female collared lizards, *Crotaphytus collaris*, stimulates courtship by males. — Herpetologica 60: 337-348.
- Beeching, S.C. (1995). Color pattern and inhibition of aggression in the cichlid fish *Astronotus ocellatus*. — J. Fish Biol. 47: 50-58.
- Bendich, A. (1989). Carotenoids and the immune-response. — J. Nutr. 119: 112-115.
- Bendich, A. & Olson, J.A. (1989). Biological actions of carotenoids. — FASEB J. 3: 1927-1932.
- Berglund, A., Bisazza, A. & Pilastro, A. (1996). Armaments and ornaments: an evolutionary explanation of traits of dual utility. — Biol. J. Linn. Soc. 58: 385-399.
- Berthouly, A., Helfenstein, F. & Richner, H. (2007). Cellular immune response, stress resistance and competitiveness in nestling great tits in relation to maternally transmitted carotenoids. — Funct. Ecol. 21: 335-343.
- Bertrand, S., Alonso-Alvarez, C., Devevey, G., Faivre, B., Prost, J. & Sorci, G. (2006a). Carotenoids modulate the trade-off between egg production and resistance to oxidative stress in zebra finches. — Oecologia 147: 576-584.
- Bertrand, S., Faivre, B. & Sorci, G. (2006b). Do carotenoid-based sexual traits signal the availability of non-pigmentary antioxidants? — J. Exp. Biol. 209: 4414-4419.
- Bezzerides, A.L., McGraw, K.J., Parker, R.S. & Husseini, J. (2007). Elytra color as a signal of chemical defense in the Asian ladybird beetle *Harmonia axyridis*. — Behav. Ecol. Sociobiol. 61: 1401-1408.

- Biard, C., Surai, P.F. & Møller, A.P. (2006). Carotenoid availability in diet and phenotype of blue and great tit nestlings. — *J. Exp. Biol.* 209: 1004-1015.
- Bjerkeng, B., Johnsen, K., Mayer, I., Storebakken, T. & Nilssen, K.J. (1999). Influence of 11-ketotestosterone, 17 beta-estradiol, and 3,5,3'-triiodo-L-thyronine on distribution and metabolism of carotenoids in Arctic charr, *Salvelinus alpinus* L. — *Fish Physiol. Biochem.* 21: 353-364.
- Bjerkeng, B., Storebakken, T. & Liaaen-Jensen, S. (1992). Pigmentation of rainbow-trout from start feeding to sexual-maturation. — *Aquaculture* 108: 333-346.
- Bjørnland, T. (1997). UV-vis spectroscopy of carotenoids. — In: *Phytoplankton pigments in oceanography*. UNESCO, Paris, p. 578-594.
- Blas, J., Perez-Rodriguez, L., Bortolotti, G.R., Vinuela, J. & Marchant, T.A. (2006). Testosterone increases bioavailability of carotenoids: insights into the honesty of sexual signaling. — *Proc. Natl. Acad. Sci. USA* 103: 18633-18637.
- Blount, J.D. (2004). Carotenoids and life-history evolution in animals. — *Arch. Biochem. Biophys.* 430: 10-15.
- Blount, J.D., Houston, D.C. & Møller, A.P. (2000). Why egg yolk is yellow. — *Trends Ecol. Evol.* 15: 131.
- Blount, J.D. & McGraw, K.J. (2008). Signal functions of carotenoid colouration. — In: *Carotenoids, Vol. 4: natural functions* (Britton, G., Liaaen-Jensen, S. & Pfander, H., eds). Birkhäuser, Basel, p. 213-236.
- Blount, J.D., Metcalfe, N.B., Birkhead, T.R. & Surai, P.F. (2003). Carotenoid modulation of immune function and sexual attractiveness in zebra finches. — *Science* 300: 125-127.
- Blount, J.D., Surai, P.F., Houston, D.C. & Møller, A.P. (2002). Patterns of yolk enrichment with dietary carotenoids in gulls: the roles of pigment acquisition and utilization. — *Funct. Ecol.* 16: 445-453.
- Bowmaker, J.K. (1995). The visual pigments of fish. — *Progr. Retinal Eye Res.* 15: 1-31.
- Britton, G. (1995). Structure and properties of carotenoids in relation to function. — *FASEB J.* 9: 1551-1558.
- Britton, G. (2008). Functions of intact carotenoids. — In: *Carotenoids, Vol. 4: natural functions* (Britton, G., Liaaen-Jensen, S. & Pfander, H., eds). Birkhäuser, Basel, p. 189-212.
- Britton, G., Liaaen-Jensen, S., Pfander, H., Mercadante, A.Z. & Egeland, E.S. (2004). *Carotenoids handbook*. — Birkhäuser, Basel.
- Brush, A.H. (1990). Metabolism of carotenoid-pigments in birds. — *FASEB J.* 4: 2969-2977.
- Burton, G.W. (1989). Antioxidant action of carotenoids. — *J. Nutr.* 119: 109-111.
- Burton, G.W. & Ingold, K.U. (1984). Beta-carotene — an unusual type of lipid antioxidant. — *Science* 224: 569-573.
- Byron, E.R. (1982). The adaptive significance of calanoid copepod pigmentation — a comparative and experimental analysis. — *Ecology* 63: 1871-1886.
- Candolin, U. (2000a). Changes in expression and honesty of sexual signalling over the reproductive lifetime of sticklebacks. — *Proc. Roy. Soc. Lond. B: Biol.* 267: 2425-2430.
- Candolin, U. (2000b). Increased signalling effort when survival prospects decrease: male-male competition ensures honesty. — *Anim. Behav.* 60: 417-422.
- Candolin, U. (2003). The use of multiple cues in mate choice. — *Biol. Rev.* 78: 575-595.
- Casagrande, S., Dijkstra, C., Tagliavini, J., Goerlich, V.C. & Groothuis, T.G. (2010). Differential effects of testosterone, dihydrotestosterone and estradiol on carotenoid deposition in an avian sexually selected signal. — *J. Comp. Physiol. A: in press* (doi: 10.1007/s00359-010-0579-4).

- Catoni, C., Peters, A. & Schaefer, H.M. (2008). Life history trade-offs are influenced by the diversity, availability and interactions of dietary antioxidants. — *Anim. Behav.* 76: 1107-1119.
- Chew, B.P. (1993). Role of carotenoids in the immune-response. — *J. Dairy Sci.* 76: 2804-2811.
- Chew, B.P. & Park, J.S. (2004). Carotenoid action on the immune response. — *J. Nutr.* 134: 257S-261S.
- Chien, Y.H. & Shiau, W.C. (2005). The effects of dietary supplementation of algae and synthetic astaxanthin on body astaxanthin, survival, growth, and low dissolved oxygen stress resistance of kuruma prawn, *Marsupenaeus japonicus* Bate. — *J. Exp. Mar. Biol. Ecol.* 318: 201-211.
- Christiansen, R., Glette, J., Lie, O., Torrissen, O.J. & Waagbo, R. (1995). Antioxidant status and immunity in atlantic salmon, *Salmo salar* L., fed semi-purified diets with and without astaxanthin supplementation. — *J. Fish Dis.* 18: 317-328.
- Cianci, M., Rizkallah, P.J., Olczak, A., Raftery, J., Chayen, N.E., Zagalsky, P.F. & Helliwell, J.R. (2002). The molecular basis of the coloration mechanism in lobster shell: beta-carustacyanin at 3.2-angstrom resolution. — *Proc. Natl. Acad. Sci. USA* 99: 9795-9800.
- Clotfelter, E.D., Ardia, D.R. & McGraw, K.J. (2007). Red fish, blue fish: trade-offs between pigmentation and immunity in *Betta splendens*. — *Behav. Ecol.* 18: 1139-1145.
- Cogdell, R.J. (1985). Carotenoids in photosynthesis. — *Pure Appl. Chem.* 57: 723-728.
- Collins, S.A. & ten Cate, C. (1996). Does beak colour affect female preference in zebra finches? — *Anim. Behav.* 52: 105-112.
- Conolly, R.B. & Lutz, W.K. (2004). Nonmonotonic dose-response relationships: mechanistic basis, kinetic modeling, and implications for risk assessment. — *Toxicol. Sci.* 77: 151-157.
- Costantini, D. (2008). Oxidative stress in ecology and evolution: lessons from avian studies. — *Ecol. Lett.* 11: 1238-1251.
- Costantini, D., Coluzza, C., Fanfani, A. & Dell'Omo, G. (2007). Effects of carotenoid supplementation on colour expression, oxidative stress and body mass in rehabilitated captive adult kestrels (*Falco tinnunculus*). — *J. Comp. Physiol. B* 177: 723-731.
- Costantini, D. & Dell'Omo, G. (2006). Effects of T-cell-mediated immune response on avian oxidative stress. — *Comp. Biochem. Physiol. A* 145: 137-142.
- Costantini, D., Fanfani, A. & Dell'Omo, G. (2008). Effects of corticosteroids on oxidative damage and circulating carotenoids in captive adult kestrels (*Falco tinnunculus*). — *J. Comp. Physiol. B* 178: 829-835.
- Costantini, D. & Møller, A.P. (2008). Carotenoids are minor antioxidants for birds. — *Funct. Ecol.* 22: 367-370.
- Craig, J.K. & Foote, C.J. (2001). Countergradient variation and secondary sexual color: phenotypic convergence promotes genetic divergence in carotenoid use between sympatric anadromous and nonanadromous morphs of sockeye salmon (*Oncorhynchus nerka*). — *Evolution* 55: 380-391.
- Craik, J.C.A. (1985). Egg quality and egg pigment content in salmonid fishes. — *Aquaculture* 47: 61-88.
- Cucco, M., Guasco, B., Malacarne, G. & Ottonelli, R. (2007). Effects of beta-carotene on adult immune condition and antibacterial activity in the eggs of the Grey Partridge, *Perdix perdix*. — *Comp. Biochem. Physiol. A* 147: 1038-1046.

- Dauwe, T. & Eens, M. (2008). Melanin- and carotenoid-dependent signals of great tits (*Parus major*) relate differently to metal pollution. — Naturwissenschaften 95: 969-973.
- Dijkstra, P.D., Hekman, R., Schulz, R.W. & Groothuis, T.G.G. (2007). Social stimulation, nuptial colouration, androgens and immunocompetence in a sexual dimorphic cichlid fish. — Behav. Ecol. Sociobiol. 61: 599-609.
- Dowling, D.K. & Simmons, L.W. (2009). Reactive oxygen species as universal constraints in life-history evolution. — Proc. Roy. Soc. Lond. B: Biol. 276: 1737-1745.
- Eberhardt, M.K. (2000). Antioxidants. — In: Reactive oxygen metabolites: chemistry and medical consequences. CRC Press, Boca Raton, FL, p. 591.
- Edge, R., McGarvey, D.J. & Truscott, T.G. (1997). The carotenoids as anti-oxidants — a review. — J. Photochem. Photobiol. B 41: 189-200.
- Elmer, K.R., Lehtonen, T. & Meyer, A. (2009). Color assortative mating contributes to sympatric divergence of neotropical cichlid fish. — Evolution 63: 2750-2757.
- Endler, J.A. (1978). A predator's view of animal color patterns. — In: Evolutionary biology (Hecht, M.K., Steere, W.C. & Wallace, B., eds). Plenum, New York, NY, p. 319-364.
- Endler, J.A. (1980). Natural-selection on color patterns in *Poecilia reticulata*. — Evolution 34: 76-91.
- Endler, J.A. (1983). Natural and sexual selection on color patterns in poeciliid fishes. — Environ. Biol. Fish. 9: 173-190.
- Evans, M.R. & Norris, K. (1996). The importance of carotenoids in signaling during aggressive interactions between male firemouth cichlids (*Cichlasoma meeki*). — Behav. Ecol. 7: 1-6.
- Ewen, J.G., Thorogood, R., Karadas, F. & Cassey, P. (2008). Condition dependence of nestling mouth colour and the effect of supplementing carotenoids on parental behaviour in the hihi (*Notiomystis cincta*). — Oecologia 157: 361-368.
- Ewen, J.G., Thorogood, R., Karadas, F., Pappas, A.C. & Surai, P.F. (2006). Influences of carotenoid supplementation on the integrated antioxidant system of a free living endangered passerine, the hihi (*Notiomystis cincta*). — Comp. Biochem. Physiol. A 143: 149-154.
- Faivre, B., Gregoire, A., Preault, M., Cezilly, F. & Sorci, G. (2003). Immune activation rapidly mirrored in a secondary sexual trait. — Science 300: 103.
- Feltl, L., Pacáková, V., Stulik, K. & Volka, K. (2005). Reliability of carotenoid analyses: a review. — Curr. Anal. Chem. 1: 93-102.
- Fenoglio, S., Cucco, M. & Malacarne, G. (2002). Bill colour and body condition in the Moorhen *Gallinula chloropus*. — Bird Stud. 49: 89-92.
- Fenoglio, S., Cucco, M. & Malacarne, G. (2003). The effect of a carotenoid-rich diet on immunocompetence and behavioural performances in Moorhen chicks. — Ethol. Ecol. Evol. 14: 149-156.
- Finkel, T. & Holbrook, N.J. (2000). Oxidants, oxidative stress and the biology of ageing. — Nature 408: 239-247.
- Fitze, P.S. & Tschirren, B. (2006). No evidence for survival selection on carotenoid-based nestling coloration in great tits (*Parus major*). — J. Evol. Biol. 19: 618-624.
- Folstad, I. & Karter, A.J. (1992). Parasites, bright males, and the immunocompetence handicap. — Am. Nat. 139: 603-622.
- Foote, C.J., Brown, G.S. & Hawryshyn, C.W. (2004). Female colour and male choice in sockeye salmon: implications for the phenotypic convergence of anadromous and nonanadromous morphs. — Anim. Behav. 67: 69-83.

- Forstmeier, W., Segelbacher, G., Mueller, J.C. & Kempenaers, B. (2007). Genetic variation and differentiation in captive and wild zebra finches (*Taeniopygia guttata*). — *Mol. Ecol.* 16: 4039-4050.
- George, S.B., Lawrence, J.M., Lawrence, A.L., Smiley, J. & Plank, L. (2001). Carotenoids in the adult diet enhance egg and juvenile production in the sea urchin *Lytechinus variegatus*. — *Aquaculture* 199: 353-369.
- Getty, T. (1998a). Handicap signalling: when fecundity and viability do not add up. — *Anim. Behav.* 56: 127-130.
- Getty, T. (1998b). Reliable signalling need not be a handicap — commentary. — *Anim. Behav.* 56: 253-255.
- Getty, T. (2002). Signaling health versus parasites. — *Am. Nat.* 159: 363-371.
- Getty, T. (2006). Sexually selected signals are not similar to sports handicaps. — *Trends Ecol. Evol.* 21: 83-88.
- Glover, M., Morton, R.A. & Rosen, D.G. (1952). Astaxanthin, cholesterol and lipins in developing salmon eggs. — *Biochem. J.* 50: 425-429.
- Godin, J.G.J. & McDonough, H.E. (2003). Predator preference for brightly colored males in the guppy: a viability cost for a sexually selected trait. — *Behav. Ecol.* 14: 194-200.
- Goodwin, T.W. (1984). The biochemistry of the carotenoids, II. Animals. — Chapman & Hall, London.
- Götmark, F. & Ahlström, M. (1997). Parental preference for red mouth of chicks in a songbird. — *Proc. Roy. Soc. Lond. B: Biol.* 264: 959-962.
- Götmark, F. & Olsson, J. (1997). Artificial colour mutation: do red-painted great tits experience increased or decreased predation? — *Anim. Behav.* 53: 83-91.
- Grafen, A. (1984). Natural selection, kin selection and group selection. — In: *Behavioural ecology*, 2nd edn. (Krebs, J.R. & Davies, N.B., eds). Blackwell Scientific Publications, Oxford, p. 62-84.
- Grafen, A. (1990a). Biological signals as handicaps. — *J. Theor. Biol.* 144: 517-546.
- Grafen, A. (1990b). Sexual selection unhandicapped by the Fisher process. — *J. Theor. Biol.* 144: 473-516.
- Grether, G.F., Cummings, M.E. & Hudon, J. (2005). Countergradient variation in the sexual coloration of guppies (*Poecilia reticulata*): drosoprotein synthesis balances carotenoid availability. — *Evolution* 59: 175-188.
- Grether, G.F., Hudon, J. & Endler, J.A. (2001). Carotenoid scarcity, synthetic pteridine pigments and the evolution of sexual coloration in guppies (*Poecilia reticulata*). — *Proc. Roy. Soc. Lond. B: Biol.* 268: 1245-1253.
- Grether, G.F., Kasahara, S., Kolluru, G.R. & Cooper, E.L. (2004a). Sex-specific effects of carotenoid intake on the immunological response to allografts in guppies (*Poecilia reticulata*). — *Proc. Roy. Soc. Lond. B: Biol.* 271: 45-49.
- Grether, G.F., Kolluru, G.R. & Nersissian, K. (2004b). Individual colour patches as multi-component signals. — *Biol. Rev.* 79: 583-610.
- Griffith, S.C., Parker, T.H. & Olson, V.A. (2006). Melanin- versus carotenoid-based sexual signals: is the difference really so black and red? — *Anim. Behav.* 71: 749-763.
- Griggio, M., Serra, L., Licheri, D., Monti, A. & Pilastro, A. (2007). Armaments and ornaments in the rock sparrow: a possible dual utility of a carotenoid-based feather signal. — *Behav. Ecol. Sociobiol.* 61: 423-433.
- Guilford, T. & Dawkins, M.S. (1991). Receiver psychology and the evolution of animal signals. — *Anim. Behav.* 42: 1-14.

- Hager, S.B. (2001). The role of nuptial coloration in female *Holbrookia maculata*: evidence for a dual signaling system. — *J. Herpetol.* 35: 624-632.
- Halliwell, B. & Gutteridge, J.M.C. (2007). *Free radicals in biology and medicine*, 4th edn. — Clarendon Press, Oxford.
- Hamilton, W.D. & Brown, S.P. (2001). Autumn tree colours as a handicap signal. — *Proc. Roy. Soc. Lond. B: Biol.* 268: 1489-1493.
- Handelman, G.J. (1996). Carotenoids as scavengers of active oxygen species. — In: *Handbook of antioxidants*. Marcel Dekker, New York, NY, p. 259-314.
- Handelman, G.J. (2001). [The evolving role of carotenoids in human biochemistry](#). — *Nutrition* 17: 818-822.
- Harper, D.G.C. (2006). Maynard Smith: amplifying the reasons for signal reliability. — *J. Theor. Biol.* 239: 203-209.
- Hartley, R.C. & Kennedy, M.W. (2004). Are carotenoids a red herring in sexual display? — *Trends Ecol. Evol.* 19: 353-354.
- Hasson, O. (1990). The role of amplifiers in sexual selection — an integration of the amplifying and the fisherian mechanisms. — *Evol. Ecol.* 4: 277-289.
- Hasson, O. (2000). Knowledge, information, biases and signal assemblages. — In: *Animal signals. Signalling and signal design in animal communication* (Espmark, Y., Amundsen, T. & Rosenqvist, G., eds). The Royal Norwegian Society of Sciences and Letters and The Foundation of Tapir Publishers, Trondheim, p. 445-463.
- Hatlen, B., Jobling, M. & Bjerkeng, B. (1998). Relationships between carotenoid concentration and colour of filets of Arctic charr, *Salvelinus alpinus* (L.), fed astaxanthin. — *Aquacult. Res.* 29: 191-202.
- Healey, M. & Olsson, M. (2009). Too big for his boots: are social costs keeping condition-dependent status signalling honest in an Australian lizard? — *Austr. Ecol.* 34: 636-640.
- Helfenstein, F., Berthouly, A., Tanner, M., Karadas, F. & Richner, H. (2008). Nestling begging intensity and parental effort in relation to prelaying carotenoid availability. — *Behav. Ecol.* 19: 108-115.
- Hill, G.E. (1991). [Plumage coloration is a sexually selected indicator of male quality](#). — *Nature* 350: 337-339.
- Hill, G.E. (1999a). Is there an immunological cost to [carotenoid-based ornamental coloration?](#) — *Am. Nat.* 154: 589-595.
- Hill, G.E. (1999b). Mate choice, male quality, and carotenoid-based plumage colouration. — In: *Proceedings of the 22nd International Ornithological Congress* (Adams, N.J. & Slotow, R.H., eds). Birdlife, Durban, p. 1654-1668.
- Hill, G.E. (2006). Environmental regulation of ornamental coloration. — In: *Bird coloration, Vol. 1: mechanisms and measurements*. Harvard University Press, London, p. 178-242.
- Hofmann, C.M., Cronin, T.W. & Omland, K.E. (2006). Using spectral data to reconstruct evolutionary changes in coloration: carotenoid color evolution in new world orioles. — *Evolution* 60: 1680-1691.
- Hofmann, C.M., Cronin, T.W. & Omland, K.E. (2007). Melanin coloration in New World orioles II: ancestral state reconstruction reveals lability in the use of carotenoids and pheomelanins. — *J. Avian Biol.* 38: 172-181.
- Hörak, P. & Saks, L. (2003). [Animal allure and health linked by plant pigments](#). — *Bioessays* 25: 746-747.
- Hörak, P., Saks, L., Zilmer, M., Karu, U. & Zilmer, K. (2007). Notes and comments — do dietary antioxidants alleviate the cost of immune activation? An experiment with greenfinches. — [Am. Nat.](#) 170: 625-635.

- Hörak, P., Sild, E., Soomets, U., Sepp, T. & Kilk, K. (2010). Oxidative stress and information content of black and yellow plumage coloration: an experiment with greenfinches. — *J. Exp. Biol.* 213: 2225-2233.
- Hörak, P., Surai, P.F., Ots, I. & Møller, A.P. (2004). Fat soluble antioxidants in brood-rearing great tits *Parus major*: relations to health and appearance. — *J. Avian Biol.* 35: 63-70.
- Hörak, P., Zilmer, M., Saks, L., Ots, I., Karu, U. & Zilmer, K. (2006). Antioxidant protection, carotenoids and the costs of immune challenge in greenfinches. — *J. Exp. Biol.* 209: 4329-4338.
- Houde, A.E. (1997). Sex, color, and mate choice in guppies. — Princeton University Press, Princeton, NJ.
- Houde, A.E. & Torio, A.J. (1992). Effect of parasitic infection on male color pattern and female choice in guppies. — *Behav. Ecol.* 3: 346-351.
- Huggins, K.A., Navara, K.J., Mendonca, M.T. & Hill, G.E. (2010). Detrimental effects of carotenoid pigments: the dark side of bright coloration. — *Naturwissenschaften* 97: 637-644.
- Hurst, J.S., Saini, M.K., Jin, G.F., Awasthi, Y.C. & van Kuijk, F. (2005). Toxicity of oxidized beta-carotene to cultured human cells. — *Exp. Eye Res.* 81: 239-243.
- Isaksson, C., McLaughlin, P., Monaghan, P. & Andersson, S. (2007). Carotenoid pigmentation does not reflect total non-enzymatic antioxidant activity in plasma of adult and nestling great tits, *Parus major*. — *Funct. Ecol.* 21: 1123-1129.
- Jayasooriya, A.P., Weisinger, R.S., Weisinger, H.S., Mathai, M.L. & Sinclair, A.J. (2002). Attraction to orange: sexiness, not gluttony. — *Science* 296: 847-847.
- Johnstone, R.A. (1996). Multiple displays in animal communication: 'backup signals' and 'multiple messages'. — *Philos. Trans. Roy. Soc. Lond. Biol.* 351: 329-338.
- Kalariya, N.M., Ramana, K.V., Srivastava, S.K. & van Kuijk, F. (2008). Carotenoid derived aldehydes-induced oxidative stress causes apoptotic cell death in human retinal pigment epithelial cells. — *Exp. Eye Res.* 86: 70-80.
- Karadas, F., Pappas, A.C., Surai, P.F. & Speake, B.K. (2005). Embryonic development within carotenoid-enriched eggs influences the post-hatch carotenoid status of the chicken. — *Comp. Biochem. Physiol. B* 141: 244-251.
- Karu, U., Saks, L. & Hörak, P. (2008). Carotenoid-based plumage coloration is not affected by vitamin E supplementation in male greenfinches. — *Ecol. Res.* 23: 931-935.
- Kiere, L.M., Hofmann, C.M., Price, J.J., Cronin, T.W. & Omland, K.E. (2009). Discrete evolutionary color changes in caciques suggest different modes of carotenoid evolution between closely related taxa. — *J. Avian Biol.* 40: 605-613.
- Kodric-Brown, A. (1989). Dietary carotenoids and male mating success in the guppy — an environmental component to female choice. — *Behav. Ecol. Sociobiol.* 25: 393-401.
- Kodric-Brown, A. (1998). Sexual dichromatism and temporary color changes in the reproduction of fishes. — *Am. Zool.* 38: 70-81.
- Kolm, N. (2004). Female courtship in the Banggai cardinalfish: honest signals of egg maturity and reproductive output? — *Behav. Ecol. Sociobiol.* 56: 59-64.
- Kotiaho, J.S. (2001). Costs of sexual traits: a mismatch between theoretical considerations and empirical evidence. — *Biol. Rev.* 76: 365-376.
- Koutsos, E.A., Calvert, C.C. & Klasing, K.C. (2003). The effect of an acute phase response on tissue carotenoid levels of growing chickens (*Gallus gallus domesticus*). — *Comp. Biochem. Physiol. A* 135: 635-646.

- Krinsky, N.I. (1974). Singlet excited oxygen as a mediator of antibacterial action of leukocytes. — Science 186: 363-365.
- Krinsky, N.I. (1989). Antioxidant functions of carotenoids. — *Free Radic. Biol. Med.* 7: 617-635.
- Krinsky, N.I. (1993). Actions of carotenoids in biological systems. — *Annu. Rev. Nutr.* 13: 561-587.
- Kurtz, J., Kalbe, M., Langefors, S., Mayer, I., Milinski, M. & Hasselquist, D. (2007). An experimental test of the immunocompetence handicap hypothesis in a teleost fish: 11-ketotestosterone suppresses innate immunity in three-spined sticklebacks. — *Am. Nat.* 170: 509-519.
- Kwiatkowski, M.A. & Sullivan, B.K. (2002). Geographic variation in sexual selection among populations of an iguanid lizard, *Sauromalus obesus* (= *ater*). — Evolution 56: 2039-2051.
- Lehtonen, T., Wong, B.B.M. & Lindström, K. (2009). Fluctuating mate preferences in a marine fish. — *Biol. Lett.* 6: 21-23.
- Liñán-Cabello, M.A., Paniagua-Michel, J. & Hopkins, P.M. (2002). Bioactive roles of carotenoids and retinoids in crustaceans. — Aquacult. Nutr. 8: 299-309.
- Lindstedt, C., Lindström, L. & Mappes, J. (2009). Thermoregulation constrains effective warning signal expression. — Evolution 63: 469-478.
- Loiseau, C., Fellous, S., Haussy, C., Chastel, O. & Sorci, G. (2008). Condition-dependent effects of corticosterone on a carotenoid-based begging signal in house sparrows. — *Horm. Behav.* 53: 266-273.
- Lozano, G.A. (1994). Carotenoids, parasites, and sexual selection. — *Oikos* 70: 309-311.
- Lozano, G.A. (2001). Carotenoids, immunity, and sexual selection: comparing apples and oranges? — *Am. Nat.* 158: 200-203.
- Lubzens, E., Lissauer, L., Levavi-Sivan, B., Avarre, J.-C. & Sammar, M. (2003). Carotenoid and retinoid transport to fish oocytes and eggs: what is the role of retinol binding protein? — *Mol. Aspects Med.* 24: 441-457.
- Macedonia, J.M., James, S., Wittle, L.W. & Clark, D.L. (2000). Skin pigments and coloration in the Jamaican radiation of *Anolis* lizards. — *J. Herpetol.* 34: 99-109.
- Maden, M. (1993). The effect of vitamin-A (retinoids) on pattern-formation implies a uniformity of developmental mechanisms throughout the animal kingdom. — *Acta Biotheor.* 41: 425-445.
- Martin, H.D., Jager, C., Ruck, C., Schmidt, M., Walsh, R. & Paust, J. (1999). Anti- and prooxidant properties of carotenoids. — *J. Prakt. Chem.* 341: 302-308.
- Matson, K.D., Cohen, A.A., Klasing, K.C., Ricklefs, R.E. & Scheuerlein, A. (2006). No simple answers for ecological immunology: relationships among immune indices at the individual level break down at the species level in waterfowl. — *Proc. Roy. Soc. Lond. B: Biol.* 273: 815-822.
- Maynard Smith, J. & Harper, D. (2003). *Animal signals*. — Oxford University Press, Oxford.
- Maynard Smith, J. & Harper, D.G.C. (1995). *Animal signals: models and terminology*. — *J. Theor. Biol.* 177: 305-311.
- McGraw, K.J. (2005). The antioxidant function of many animal pigments: are there consistent health benefits of sexually selected colourants? — *Anim. Behav.* 69: 757-764.
- McGraw, K.J. (2006a). Dietary carotenoids mediate a trade-off between egg quantity and quality in Japanese quail. — *Ethol. Ecol. Evol.* 18: 247-256.

- McGraw, K.J. (2006b). Mechanics of carotenoid-based coloration. — In: Bird coloration, Vol. 1: mechanisms and measurements (Hill, G.E., ed.). Harvard University Press, London: p. 178-242.
- McGraw, K.J. (2006c). Sex steroid dependence of carotenoid-based coloration in female zebra finches. — *Physiol. Behav.* 88: 347-352.
- McGraw, K.J. (2007). Do carotenoids buffer testosterone-induced immunosuppression? An experimental test in a colourful songbird. — *Biol. Lett.* 3: 375-378.
- McGraw, K.J. & Ardia, D.R. (2003). Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. — *Am. Nat.* 162: 704-712.
- McGraw, K.J., Correa, S.M. & Adkins-Regan, E. (2006a). Testosterone upregulates lipoprotein status to control sexual attractiveness in a colorful songbird. — *Behav. Ecol. Sociobiol.* 60: 117-122.
- McGraw, K.J., Crino, O.L., Medina-Jerez, W. & Nolan, P.M. (2006b). Effect of dietary carotenoid supplementation on food intake and immune function in a songbird with no carotenoid coloration. — *Ethology* 112: 1209-1216.
- McGraw, K.J. & Klasing, K.C. (2006). Carotenoids, immunity, and integumentary coloration in red junglefowl (*Gallus gallus*). — *Auk* 123: 1161-1171.
- McKinnon, J.S., Demayo, R.F., Granquist, R. & Weggel, L. (2000). Female red throat coloration in two populations of threespine stickleback. — *Behaviour* 137: 947-963.
- Miki, W. (1991). Biological functions and activities of animal carotenoids. — *Pure Appl. Chem.* 63: 141-146.
- Milinski, M. & Bakker, T.C.M. (1990). Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. — *Nature* 344: 330-333.
- Miller, N.J., Sampson, J., Candeias, L.P., Bramley, P.M. & Rice-Evans, C.A. (1996). Antioxidant activities of carotenes and xanthophylls. — *FEBS Lett.* 384: 240-242.
- Møller, A.P., Biard, C., Blount, J.D., Houston, D.C., Ninni, P., Saino, N. & Surai, P.F. (2000). Carotenoid-dependent signals: indicators of foraging efficiency, immunocompetence or detoxification ability? — *Avian Poultry Biol. Rev.* 11: 137-159.
- Møller, A.P. & Pomiankowski, A. (1993). Why have birds got multiple sexual ornaments. — *Behav. Ecol. Sociobiol.* 32: 167-176.
- Monaghan, P., Metcalfe, N.B. & Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. — *Ecol. Lett.* 12: 75-92.
- Moore, T. (1930). VI. The conversion of carotene to vitamin A in vivo. — *Biochem. J.* 24: 696-702.
- Morales, J., Velando, A. & Torres, R. (2008). Fecundity compromises attractiveness when pigments are scarce. — *Behav. Ecol.* 20: 117-123.
- Morton, R.A. & Creed, R.H. (1939). The conversion of carotene to vitamin A₂ by some fresh-water fishes. — *Biochem. J.* 33: 318-324.
- Mougeot, F., Perez-Rodriguez, L., Martinez-Padilla, J., Leckie, F. & Redpath, S.M. (2007). Parasites, testosterone and honest carotenoid-based signalling of health. — *Funct. Ecol.* 21: 886-898.
- Nordeide, J.T., Mohus, A., Nicolaisen, O., Volden, R. & Egeland, E.S. (2008). Offspring or ornaments? Is carotenoid-based ornamentation in female Arctic charr, *Salvelinus alpinus* (L.), condition-dependent and traded off against offspring? — *Ecol. Freshw. Fish* 17: 328-339.

- Nys, Y. (2000). Dietary carotenoids and egg yolk coloration — a review. — Arch. Gefügelk. 64: 45-54.
- Oakes, K.D. & Van der Kraak, G.J. (2003). Utility of the TBARS assay in detecting oxidative stress in white sucker (*Catostomus commersoni*) populations exposed to pulp mill effluent. — Aquat. Toxicol. 63: 447-463.
- Okimasu, E., Matsumoto, M., Yoshida, Y. & Amemura, A. (1992). The effect of pigments of *Rhodobacter capsulatus* on free-radicals and application of the bacterium as feed to fish larvae. — Nippon Suisan Gakkai Shi 58: 1487-1491.
- Olson, V.A. & Owens, I.P.F. (1998). Costly sexual signals: are carotenoids rare, risky or required? — Trends Ecol. Evol. 13: 510-514.
- Olson, V.A. & Owens, I.P.F. (2005). Interspecific variation in the use of carotenoid-based coloration in birds: diet, life history and phylogeny. — J. Evol. Biol. 18: 1534-1546.
- Olsson, M., Healey, M., Wapstra, E., Schwartz, T., Lebas, N. & Uller, T. (2007). Mating system variation and morph fluctuations in a polymorphic lizard. — Mol. Ecol. 16: 5307-5315.
- Østerlie, M. (2000). Nutritional aspects of astaxanthin isomers. — PhD thesis, Department of Chemistry, NTNU, Trondheim.
- Østerlie, M., Bjerkeng, B. & Liaaen-Jensen, S. (1999). Accumulation of astaxanthin all-E, 9Z and 13Z geometrical isomers and 3 and 3' RS optical isomers in rainbow trout (*Oncorhynchus mykiss*) is selective. — J. Nutr. 129: 391-398.
- Palmer, L.S. & Kempster, H.L. (1919). The influence of specific feeds and certain pigments on the color of the egg yolk and body fat of fowls. — J. Biol. Chem. 39: 331-337.
- Palozza, P. (1998). Prooxidant actions of carotenoids in biologic systems. — Nutr. Rev. 56: 257-265.
- Parker, R.S. (1996). Carotenoids. 4. Absorption, metabolism, and transport of carotenoids. — FASEB J. 10: 542-551.
- Perez-Rodriguez, L. (2009). Carotenoids in evolutionary ecology: re-evaluating the antioxidant role. — Bioessays 31: 1116-1126.
- Perez-Rodriguez, L., Mougeot, F., Alonso-Alvarez, C., Blas, J., Vinuela, J. & Bortolotti, G.R. (2008). Cell-mediated immune activation rapidly decreases plasma carotenoids but does not affect oxidative stress in red-legged partridges (*Alectoris rufa*). — J. Exp. Biol. 211: 2155-2161.
- Perez, C., Lores, M. & Velando, A. (2008). Availability of nonpigmentary antioxidant affects red coloration in gulls. — Behav. Ecol. 19: 967-973.
- Peters, A. (2007). Testosterone and carotenoids: an integrated view of trade-offs between immunity and sexual signalling. — Bioessays 29: 427-430.
- Pike, T.W., Blount, J.D., Bjerkeng, B., Lindström, J. & Metcalfe, N.B. (2007a). Carotenoids, oxidative stress and female mating preference for longer lived males. — Proc. Roy. Soc. Lond. B: Biol. 274: 1591-1596.
- Pike, T.W., Blount, J.D., Lindström, J. & Metcalfe, N.B. (2007b). Availability of non-carotenoid antioxidants affects the expression of a carotenoid-based sexual ornament. — Biol. Lett. 3: 353-356.
- Pike, T.W., Blount, J.D., Lindström, J. & Metcalfe, N.B. (2007c). Dietary carotenoid availability influences a male's ability to provide parental care. — Behav. Ecol. 18: 1100-1105.
- Pryke, S.R., Andersson, S. & Lawes, M.J. (2001). Sexual selection of multiple handicaps in the red-collared widowbird: female choice of tail length but not carotenoid display. — Evolution 55: 1452-1463.

- Pryke, S.R., Andersson, S., Lawes, M.J. & Piper, S.E. (2002). Carotenoid status signaling in captive and wild red-collared widowbirds: independent effects of badge size and color. — *Behav. Ecol.* 13: 622-631.
- Rahman, M.L., Yoshida, K., Maeda, I., Tanaka, H. & Sugita, S. (2010). Distribution of retinal cone photoreceptor oil droplets, and identification of associated carotenoids in crow (*Corvus macrorhynchos*). — *Zool. Sci.* 27: 514-521.
- Rajasingh, H., Oyehaug, L., Vage, D.I. & Omholt, S.W. (2006). Carotenoid dynamics in Atlantic salmon. — *BMC Biol.* 4: 15.
- Rajasingh, H., Vage, D.I., Pavey, S.A. & Omholt, S.W. (2007). Why are salmonids pink? — *Can. J. Fish. Aquat. Sci.* 64: 1614-1627.
- Rao, A.V. & Rao, L.G. (2007). Carotenoids and human health. — *Pharmacol. Res.* 55: 207-216.
- Riechert, S.E. (1978). Games spiders play — behavioral variability in territorial disputes. — *Behav. Ecol. Sociobiol.* 3: 135-162.
- Rohwer, S. (1975). Social significance of avian winter plumage variability. — *Evolution* 29: 593-610.
- Rønnestad, I., Hemre, G.I., Finn, R.N. & Lie, O. (1998). Alternate sources and dynamics of vitamin A and its incorporation into the eyes during the early endotrophic and exotrophic larval stages of Atlantic halibut (*Hippoglossus hippoglossus* L.). — *Comp. Biochem. Physiol. A* 119: 787-793.
- Rowland, W.J., Baube, C.L. & Horan, T.T. (1991). Signaling of sexual receptivity by pigmentation pattern in female sticklebacks. — *Anim. Behav.* 42: 243-249.
- Rutkowska, J. (2007). Genealogy of zebra finch lab populations: results from call for DNA samples. — *Behav. Ecol.* 19 (Suppl.): S7.
- Saino, N., Ferrari, R., Romano, M., Martinelli, R. & Møller, A.P. (2003). Experimental manipulation of egg carotenoids affects immunity of barn swallow nestlings. — *Proc. Roy. Soc. Lond. B: Biol.* 270: 2485-2489.
- Saino, N., Ninni, P., Calza, S., Martinelli, R., De Bernardi, F. & Møller, A.P. (2000). Better red than dead: carotenoid-based mouth coloration reveals infection in barn swallow nestlings. — *Proc. Roy. Soc. Lond. B: Biol.* 267: 57-61.
- Sandre, S.L., Tammaru, T., Esperk, T., Julkunen-Tiitto, R. & Mappes, J. (2007). Carotenoid-based colour polyphenism in a moth species: search for fitness correlates. — *Entomol. Exp. Appl.* 124: 269-277.
- Schwedhelm, E., Maas, R., Troost, R. & Boger, R.H. (2003). Clinical pharmacokinetics of antioxidants and their impact on systemic oxidative stress. — *Clin. Pharmacokin.* 42: 437-459.
- Senar, J.C. (2006). Color displays as intrasexual signals of aggression — In: *Bird coloration, Vol. 2: function and evolution* (Hill, G.E., ed.). Harvard University Press, London, p. 87-136.
- Shapiro, S.S., Mott, D.J. & Machlin, L.J. (1984). Kinetic characteristics of beta-carotene uptake and depletion in rat-tissue. — *J. Nutr.* 114: 1924-1933.
- Shimidzu, N., Goto, M. & Miki, W. (1996). Carotenoids as singlet oxygen quenchers in marine organisms. — *Fish. Sci.* 62: 134-137.
- Siefermann-Harms, D. (1985). Carotenoids in photosynthesis. 1. Location in photosynthetic membranes and light-harvesting function. — *Biochim. Biophys. Acta* 811: 325-355.

- Siems, W., Wiswedel, I., Salerno, C., Crifo, C., Augustin, W., Schild, L., Langhans, C.D. & Sommerburg, O. (2005). beta-carotene breakdown products may impair mitochondrial functions — potential side effects of high-dose beta-carotene supplementation. — *J. Nutr. Biochem.* 16: 385-397.
- Sies, H. (1991). Oxidative stress: introduction. — In: *Oxidative stress: oxidants and antioxidants* (Sies, H., ed.). Academic Press, London, p. xv-xxii.
- Sköld, H.N., Amundsen, T., Svensson, P.A., Mayer, I., Bjelvenmark, J. & Forsgren, E. (2008). Hormonal regulation of female nuptial coloration in a fish. — *Horm. Behav.* 54: 549-556.
- Smith, H.G., Råberg, L., Ohlsson, T., Granbom, M. & Hasselquist, D. (2007). Carotenoid and protein supplementation have differential effects on pheasant ornamentation and immunity. — *J. Evol. Biol.* 20: 310-319.
- Steffen, J.E. & McGraw, K.J. (2007). Contributions of pterin and carotenoid pigments to dewlap coloration in two anole species. — *Comp. Biochem. Physiol. B* 146: 42-46.
- Steven, D.M. (1949). Studies on animal carotenoids. 2. Carotenoids in the reproductive cycle of the brown trout. — *J. Exp. Biol.* 26: 295-303.
- Storebakken, T. & Goswami, U.C. (1996). Plasma carotenoid concentration indicates the availability of dietary astaxanthin for Atlantic salmon, *Salmo salar*. — *Aquaculture* 146: 147-153.
- Sumner, F.B. & Fox, D.L. (1933). A study of variations in the amount of yellow pigment (xanthophyll) in certain fishes, and of the possible effects upon this of colored backgrounds. — *J. Exp. Zool.* 66: 263-301.
- Surai, P.F. (2002). *Natural antioxidants in avian nutrition and reproduction*. — Nottingham University Press, Nottingham.
- Surai, P.F. & Speake, B.K. (1998). Distribution of carotenoids from the yolk to the tissues of the chick embryo. — *J. Nutr. Biochem.* 9: 645-651.
- Surai, P.F., Speake, B.K., Noble, R.C. & Sparks, N.H.C. (1999). Tissue-specific antioxidant profiles and susceptibility to lipid peroxidation of the newly hatched chick. — *Biol. Trace Elem. Res.* 68: 63-78.
- Surai, P.F., Speake, B.K. & Sparks, N.H.C. (2001a). Carotenoids in avian nutrition and embryonic development. 2. Antioxidant properties in discrimination in embryonic tissues. — *J. Poultry Sci.* 38: 117-145.
- Surai, P.F., Speake, B.K., Wood, N.A.R., Blount, J.D., Bortolotti, G.R. & Sparks, N.H.C. (2001b). Carotenoid discrimination by the avian embryo: a lesson from wild birds. — *Comp. Biochem. Physiol. B* 128: 743-750.
- Svensson, P.A., Blount, J.D., Forsgren, E. & Amundsen, T. (2009). Female ornamentation and egg carotenoids of six sympatric gobies. — *J. Fish Biol.* 75: 2777-2787.
- Svensson, P.A., Pélabon, C., Blount, J.D., Surai, P.F. & Amundsen, T. (2006). Does female nuptial coloration reflect egg carotenoids and clutch quality in the two-spotted goby (*Gobiusculus flavescens*, Gobiidae)? — *Funct. Ecol.* 20: 689-698.
- Taylor, E.B., Foote, C.J. & Wood, C.C. (1996). Molecular genetic evidence for parallel life-history evolution within a Pacific salmon (sockeye salmon and kokanee, *Oncorhynchus nerka*). — *Evolution* 50: 401-416.
- Thorogood, R., Kilner, R.M., Karadas, F. & Ewen, J.G. (2008). Spectral mouth colour of nestlings changes with carotenoid availability. — *Funct. Ecol.* 22: 1044-1051.
- Tinbergen, N. & Perdeck, A.C. (1950). On the stimulus situation releasing the begging response in the newly hatched herring gull chick (*Larus argentatus argentatus* Pont). — *Behaviour* 3: 1-39.

- Toral, G.M., Figuerola, J. & Negro, J.J. (2008). Multiple ways to become red: pigment identification in red feathers using spectrometry. — *Comp. Biochem. Physiol. B* 150: 147-152.
- Torrissen, O.J. & Christiansen, R. (1995). Requirements for carotenoids in fish diets. — *J. Appl. Ichthyol.* 11: 225-230.
- Tschirren, B., Fitze, P.S. & Richner, H. (2005). Carotenoid-based nestling colouration and parental favouritism in the great tit. — *Oecologia* 143: 477-482.
- Tsushima, M., Kawakami, T., Mine, M. & Matsuno, T. (1997). The role of carotenoids in the development of the sea urchin *Pseudocentrotus depressus*. — *Invertebr. Reprod. Dev.* 32: 149-153.
- Vanhoutteghem, A., Londero, T., Ghinea, N. & Djian, P. (2004). Serial cultivation of chicken keratinocytes, a composite cell type that accumulates lipids and synthesizes a novel beta-keratin. — *Differentiation* 72: 123-137.
- Vinkler, M. & Albrecht, T. (2010). Carotenoid maintenance handicap and the physiology of carotenoid-based signalisation of health. — *Naturwissenschaften* 97: 19-28.
- von Frisch, K. (1967). The dance language and orientation of bees. — Harvard University Press, Cambridge, MA.
- von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D. & Wittzell, H. (1999). Good genes, oxidative stress and condition-dependent sexual signals. — *Proc. Roy. Soc. Lond. B: Biol.* 266: 1-12.
- Wathne, E., Bjerkeng, B., Storebakken, T., Vassvik, V. & Odland, A.B. (1998). Pigmentation of Atlantic salmon (*Salmo salar*) fed astaxanthin in all meals or in alternating meals. — *Aquaculture* 159: 217-231.
- Watkins, G.G. (1997). Inter-sexual signalling and the functions of female coloration in the tropidurid lizard *Microlophus occipitalis*. — *Anim. Behav.* 53: 843-852.
- Weiss, S.L. (2002). Reproductive signals of female lizards: pattern of trait expression and male response. — *Ethology* 108: 793-813.
- Wicksten, M.K. (1989). Why are there bright colors in sessile marine invertebrates? — *Bull. Mar. Sci.* 45: 519-530.
- Winston, G.W., Lemaire, D.G.E. & Lee, R.F. (2004). Antioxidants and total oxyradical scavenging capacity during grass shrimp, *Palaemonetes pugio*, embryogenesis. — *Comp. Biochem. Physiol. C* 139: 281-288.
- Yeh, S.L. & Wu, S.H. (2006). Effects of quercetin on beta-apo-8'-carotenal-induced DNA damage and cytochrome P1A2 expression in A549 cells. — *Chem.-Biol. Interact.* 163: 199-206.
- Young, A.J. & Lowe, G.M. (2001). Antioxidant and prooxidant properties of carotenoids. — *Arch. Biochem. Biophys.* 385: 20-27.
- Zagalsky, P.F., Ceccaldi, H.J. & Daumas, R. (1970). Comparative studies on some decapod crustacean carotenoproteins. — *Comp. Biochem. Physiol.* 34: 579-607.
- Zahavi, A. (1975). Mate selection — selection for a handicap. — *J. Theor. Biol.* 53: 205-214.
- Zebisch, K., Krautwald-Junghanns, M.E. & Willuhn, J. (2004). Ultrasound-guided liver biopsy in birds. — *Vet. Radiol. Ultrasound* 45: 241-246.
- Zhang, L.X., Cooney, R.V. & Bertram, J.S. (1992). Carotenoids up-regulate connexin43 gene-expression independent of their provitamin A or antioxidant properties. — *Cancer Res.* 52: 5707-5712.
- Zhao, W., Han, Y.S., Zhao, B.L., Hirota, S., Hou, J.W. & Xin, W.J. (1998). Effect of carotenoids on the respiratory burst of rat peritoneal macrophages. — *Biochim. Biophys. Acta* 1381: 77-88.