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What is the value of a yellow patch? Assessing the signalling role of yellow colouration in the European serin

Sandra Trigo · Paulo G. Mota

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Abstract Sexual selection promotes the evolution of signals, many of which can reliably indicate condition, health or good genes of individuals. In order to be evolutionarily stable, indicator signals must be costly to produce. Carotenoid colouration evolved in many species by sexual selection. Carotenoids besides acting as pigments have been implicated in immune defence and antioxidation which makes them likely candidates for honest signalling. A trade-off for carotenoid availability was proposed as the basis for signal honesty. Alternatively, it was suggested that carotenoid colouration is not advertising the presence of the pigment per se, but the quality of antioxidant resources which then affect carotenoid concentration. One possibility is that carotenoid-based colouration could signal colourless antioxidant mechanisms, which are partially regulated by vitamins. β-Carotene is one of the most common precursors of vitamin A and, although present in bird diet, is not available for feather colouration. If an indirect association exists between carotenoid signal and condition, then manipulation of β-carotene concentration could reveal that this link is indirect. We tested this by conditioning the availability of β-carotene in the diet of a cardueline finch with yellow carotenoid colouration during moult. β-Carotene-supplemented males had higher plasma carotenoid concentration and higher response to a cellular immunity challenge (phytohaemagglutinin (PHA)) than control males. β-Carotene-supplemented males also had more saturated plumage colouration and were preferred by females in a mate choice test. Our results support the possibility of an indirect role for yellow carotenoid colouration.

Keywords Carotenoid-based ornamentation · Immune response · Colouration · Sexual signals · Sexual selection

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S. Trigo (✉) · P. G. Mota
Department of Life Sciences, University of Coimbra, Apt. 3046, 3301-401 Coimbra, Portugal
e-mail: trigosandra@gmail.com

S. Trigo · P. G. Mota
CIBIO, Research Center in Biodiversity and Genetic Resources, University of Porto, Campus Agrário de Vairão, 4485-661 Vairão, Portugal

Introduction

Sexual selection is an evolutionary process that favours the evolution of a class of signals which are indicators of quality (Andersson 1994). The evolution of these signals is dependent on the existence of costs for their production and maintenance, since only signals that are costly can be 'honest' indicators of quality (Zahavi 1975; Grafen 1990; Searcy and Nowicki 2005). Carotenoid colouration is widespread among vertebrates and is frequently involved in sexual signalling (Olson and Owens 1998; Møller et al. 2000), including most of the yellow, orange and red colouration of the integuments of birds, reptiles and fishes (Olson and Owens 1998), and is widely accepted to be condition-dependent, linked to individual's ability to acquire, assimilate and process carotenoids (Hill 1990, 1999). The conspicuous plumage colouration of birds is a main example of signal evolution by sexual selection (McGraw 2006). Carotenoid colouration is one of the most widespread types of social signals in birds and one of the best studied kind of ornamental traits, being involved in sexual communication, nestling signalling and mate choice (Hõrak and Saks 2003). It was shown that females prefer to mate with males that display more intense carotenoid colouration in different avian species (reviewed in Hill 2006; and also Simons and Verhulst 2011; Toomey and McGraw 2012) and also in the serins *Serinus serinus* (Leitão et al. 2014).

Carotenoids act as pigmentary molecules of bright plumage, fleshy tissues and other bare parts, but they can also be

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stored in the liver (del Val et al. 2009) or fat depots from which they might be mobilised (Ninni et al. 2004). According to the pigment allocation hypothesis (Lozano 1994), the maintenance of honesty of sexual signals is assured by two non-mutually exclusive factors: (1) dietary carotenoids are a limiting resource (Endler 1983) and (2) carotenoids have antioxidant functions and modulate immune responses. A trade-off was assumed to exist between the use of carotenoids in ornamental colouration and in several physiological functions, which would assure the honesty of the signal (Blount 2004). These pigments are not synthesised by vertebrates, so they have to be acquired through their diet as intact macromolecules (Goodwin 1984; McGraw 2006), which can be a limiting factor through pigment availability and can contribute to the signal reliability of the animal's foraging capacity and condition. Other costs could contribute to the honesty of the signal such as those related to maintaining physical condition or to other fitness-related traits (Lozano 1994; McGraw and Ardia 2003; Blas et al. 2006; Pérez-Rodriguez et al. 2010). It is known that these pigments are health-related, enhancing immune function and antioxidant activity (Lozano 1994; Olson and Owens 1998; Blount et al. 2003; Faivre et al. 2003; reviewed in Blount 2004). This was recently supported in a meta-analysis by Simons et al. (2012). Carotenoids are thought to be responsible for enhancing immunity mediated by cells, antibody production and gene expression and for enabling protection to cells and tissues from oxidation (Chew and Park 2004). They can also inhibit mutagenesis and have a role in photoprotection (Bendich and Olson 1989; Krinsky 1989).

There is clear evidence that carotenoid availability affects colour expression and immune response. In several species of birds, carotenoid-supplemented males had higher immune responses than non-supplemented males (Fenoglio et al. 2002; McGraw and Ardia 2003, 2005; Aguilera and Amat 2007) and improved growth and survival (Saino et al. 2003; Biard et al. 2006). Also, more colourful birds had higher immune responses (Saks et al. 2003; Mougeot 2008) and experienced less oxidative stress (Pérez-Rodriguez et al. 2010). Conversely, immune activation caused a decrease in colouration and plasma carotenoid levels (Alonso-Alvarez et al. 2004; Peters et al. 2004; Baeta et al. 2008; Pérez-Rodriguez et al. 2008). However, other studies have failed to find a relationship between carotenoids and oxidative stress in vivo (El-Agamey et al. 2004; Hartley and Kennedy 2004; Costantini and Møller 2008). In addition, in high dosages, carotenoids could even have a pro-oxidant activity (Bertrand et al. 2006a; Costantini and Møller 2008; Huggins et al. 2010).

Countering the trade-off hypothesis for the honesty of carotenoid signalling, Hartley and Kennedy (2004) suggested that carotenoids might not signal directly the carotenoid antioxidant capacity but, instead, signal the quality of other antioxidant resources of the animal. Antioxidants are molecules that scavenge free radicals, thus preventing oxidative stress to damage cells (Surai 2002; Martínez et al. 2008), and these antioxidant molecules include vitamins C, E and A, and antioxidant enzymes (Hartley and Kennedy 2004). Vitamin A has a variety of functions on basic life processes, such as vision, reproduction, growth and development, and also on redox homeostasis and is obtained from animal tissues or derived from β-carotene and other pro-vitamin A carotenoids (Biesalski et al. 2007). Thus, if the availability of carotenoids which do not take part in colouration was increased, and there was both an increase in health-related functions and in colouration, then this would constitute a proof for the indirect signalling role of carotenoid colouration. β-Carotene is a powerful antioxidant molecule (Bendich 1989; Krinsky 1989; Chew 1993) and possesses immunoregulatory activities (Bendich 1989; Chew 1993; Cucco et al. 2006), and it is one of the most important vitamin A precursors (Chew 1993) and also is not involved in feather colouration. If β-carotene can protect pigmentary carotenoids from oxidation, it is possible that it also affects carotenoid uptake into feather colouration and signal expression in an indirect way.

In order to test this, we conducted a full year-round study, manipulating β-carotene availability for male European serins (*S. serinus*) during moult, testing its effect on immune and physical condition and on plumage expression, and lastly testing its effect on female choice over these males in the following breeding season. The European serin is a small social sexually dichromatic seed-eater finch (Cramp and Perrins 1994) with males exhibiting a carotenoid-based yellow plumage (Stradi et al. 1995a) which goes through a single post-reproductive moult (Pagani-Nuñez and Senar 2012). The colouration of serin feathers is the result of deposition of canary xanthophylls A and B (Stradi et al. 1995b), resulting from oxidization of dietary lutein (McGraw et al. 2001). Carotenoid colouration has been shown to be sexually selected in serins (Leitão et al. 2014) and related to survival in the wild (Figuerola and Senar 2007; Pagani-Nuñez and Senar 2012). Thus we predict that (1) β-carotene supplementation will enhance the plasma carotenoid levels, immune system and physical condition of males; (2) β-carotene supplementation will enhance the colouration of males; and (3) β-carotene-supplemented males are preferred by females in mate choice experiments.

Methods

Subjects and housing

Males were captured in the winter (months 1–2 in Fig. 1), with mist nets in agricultural fields nearby Coimbra, Portugal. Birds were ringed and housed at the Department of Life Sciences, University Coimbra, until the end of the

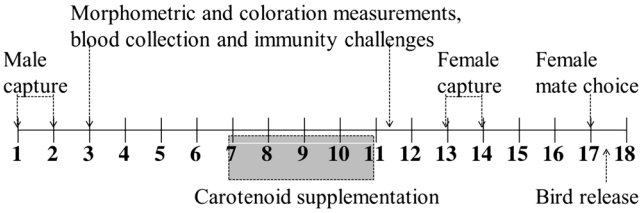

Fig. 1 Experimental timeline. Numbers are months (month 1 = January 2009)

experiments (month 17 in Fig. 1), in wired cages, under natural light and ventilation, with ad libitum access to a commercial food mixture (European Finches Prestige, Versele-Laga, composition: canary seed 46 %, rapeseed 22 %, niger seed 7 %, linseed 7 %, peeled oats 6 %, hempseed 5 %, wild seeds 5 %, radish seed 1 %, and spinach seed 1 %), tap water and commercial mix grit with oyster shell. All males had ad libitum access to the same seed mixture and had a supplement of glucose two times a week. These conditions allowed males to moult on a natural light regime. A subset of those birds was subject to mate choice tests in the spring of 2010. Morphometric and colouration measurements, blood collection and immunity challenges were made before and after the carotenoid supplementation (months 3 and 11 in Fig. 1). Physical condition was calculated as the residuals of a regression of body mass over tarsus length, a reliable and the most used estimate of condition (Jakob et al. 1996; Ots et al. 1998). The relationship between the two variables was linear, with residuals over tarsus having an even distribution (Schulte-Hostedde et al. 2005). Ectoparasite mite load on wing feathers was assessed by an estimating method following Behnke et al. (1995, 1999).

Carotenoid supplementation

Males were randomly assigned to two treatment conditions before moult: β-carotene supplementation (β-supplemented males) that received daily 0.2 g/l β-carotene (Betanat Cold Water Dispersible 1 %, Vitatene, León, Spain) diluted in water as a substitute for water and control non-supplemented males (control), which only received water. This carotenoid dosage was estimated by comparison with other studies (e.g. Navara and Hill 2003) and by a previous experiment in our laboratory. The carotenoid supplementation lasted for the entire moult period (months 7 to 11 in Fig. 1).

Measurement of carotenoid plasma concentration

Plasma carotenoid concentration was determined by transmission spectrophotometry, following a protocol that provides good estimates of plasma total carotenoid concentration, which are highly correlated with results from high-performance liquid chromatography (Alonso-Alvarez et al. 2004; Aguilera and Amat 2007). Carotenoids were quantified by diluting the plasma into 100 % acetone (1:10), vortexed for 5 s and centrifuged, at 1000 rpm for 10 min, to precipitate the flocculent protein. The absorbance of the supernatant was measured with a transmission spectrophotometer Shimadsu UV-1601, at 476 nm. The total plasma carotenoid concentration (μg/ml) was calculated using a standard curve of lutein 'α-carotene-3,3′-diol' (Sigma-Aldrich).

Tests of immunity response

Two immunity challenges were performed: the sheep red blood cell (SRBC) haemagglutination assay and the phytohaemagglutinin (PHA-P) wing web assay. SRBC antigen challenges T-dependent humoral immunity (Ots et al. 2001; Hasselquist and Nilsson 2012), and PHA-P wing web challenges the immunity mediated by cells, involving both innate and adaptive responses of the immune system (Martin et al. 2006; Tella et al. 2008). For the SRBC assay, males were inoculated, intra-abdominally, with 20 μl of 2 % SRBC in phosphate-buffered saline (PBS). A week later, about 100 μl of blood was collected from birds and centrifuged, and the plasma was preserved in −20 °C. Plasma was used to perform a haemagglutination assay using a base 2 serial dilution in a 96-well round-bottomed microtitre plate. The plate was incubated at 37 °C for 60 min, and the trite of the antibody was given by the last well with agglutination. For the PHA-P wing web test, we used a protocol following Smits et al. (1999) by measuring the wing web of males three times (with values being averaged) before inoculation. Birds were then injected in the wing web with a suspension of 20 μg PHA-P (Sigma-Aldrich L-8754, USA) in 20 μl PBS, and the wing was measured again after 24 h, following the same procedure. The intensity of response was assessed through wing swallowing between the two measurement days. We used a calliper to the nearest 0.01 mm to measure wing web thickness at the injection point. All measurements were made by the same researcher (PGM), who was unaware of birds' treatment condition, and were highly repeatable pre- and post-injection, before and after moult (r=0.74, P<0.0001, n=12; r=0.94, P<0.0001, n=12; r=0.68, P<0.0001, n=17; r=0.93, P<0.0001, n=17).

Colouration measurements

We measured the plumage colouration of males with a spectrophotometer Ocean Optics USB4000 (Ocean Optics, Dunedin, FL, USA), with deuterium and halogen light source (Mikropack Mini-DT-2-GS, UV-vis-NIR), emitting light between 300 and 700 nm, and an optical fibre reflectance probe (Ocean Optics R400-7 UV/vis), held vertically, attached to a rigid black holder to standardise the distance between probe and sample (3 mm), providing a sampling area of 28 mm^2. All measurements of the spectrum were expressed as the

proportion of light relative to a white standard (Ocean Optics, WS-1-SS White Standard). We took measurements in four different areas: forehead, throat, breast and belly, making three readings from each sampled area which were averaged. We calculated colour variables applying avian visual models, for each area, that was after averaged. Each cone quantum catch for each of the four avian cones was calculated as the summed product of plumage reflectance, ambient illuminant (standard daylight D 65), and absorbance spectrum of the cone across the entire wavelength of the avian visual spectrum (Eq. 1 in Vorobyev et al. 1998). We used the visual model of blue tit *Cyanistes caeruleus* (Hart et al. 2000), commonly used as representative of birds with UVS vision (Håstad et al. 2005). Plumage colouration was scored by two independent variables: short-wavelength-sensitive (SWS) ratio and double cone, representing chromatic and achromatic indices of plumage reflectance (Evans et al. 2010; Osorio et al. 1999), computed by software PAVO (Maia et al. 2013), running in R (R development Core Team 2013). The area of the yellow patches of the forehead and chest was measured by overlapping transparent grids and counting the number of squares covering these areas.

Mate choice experiment

Females were captured during the winter (months 13–14 in Fig. 1) and were housed in separate cages in the same facilities, but with no visual contact with males. The mate choice experiments were performed in a test room, with three compartments (main 155×272×220 cm; smaller 112×136×220 cm) (details in Leitão et al. 2014). This two-way apparatus has the best performance in this kind of test, with low estimation errors (Bruzzone and Corley 2011). The aviary apparatus had full-spectrum fluorescent lights (Philips TL950 Full Spectrum Fluorescent). During trials, a female was placed in the main compartment, facing the two males and separated from them by a glass. The two males were in adjacent compartments separated by an opaque wall. Twelve females were used in the tests performed in early spring (month 17 in Fig. 1). Each female was tested only once against a pair of males, one from each treatment group. Males were randomly assigned to each of the two compartments in order to eliminate possible female positional preferences. No combination of two males was repeated. The trials lasted 45 min, being the first 15 min considered habituation time. Tests were video-recorded, and the analysis was performed with the Observer 5 software (Noldus, Wageningen, The Netherlands). The closest area to each male's compartment in the female's compartment was designated as female's 'choice area' (see Fig. 1 in Leitão et al. 2014). We used time spent by females in the interaction area of males as a measurement of female preference (Nolan and Hill 2004 and wherein references). Males did not sing during the trials.

Statistical analysis

We analysed the differences between treatments using repeated measures ANOVAs, with treatment and time (before/after treatment) as fixed factors and including the interaction effect. When the interaction was significant, we subsequently performed one-way ANOVAs before and after the diet treatment, to test for differences between the two groups at each moment. The female mate choice tests were analysed through a generalised linear model (GLM) with repeated measures (with normal error distribution), having individual female as the subject and the female time spent in the choice area as the dependent variable and male treatment group as the within-subject factor for pairwise comparisons. To control for male behaviour, we included 'male activity' as a fixed factor. Male activity was estimated as the first component of a principal component analysis (PCA) of time in choice area and number of hops in perches (which explained 57.1 % of variation, with positive loads for both variables). The Wald χ^2 statistic was used to test for significance in the GLMs. Sample sizes were not equal for all measured variables, due to problems with blood collection or insufficient plasma volume. So, we report sample sizes in all analysis. All statistical analyses were performed with IBM SPSS Statistics 19.0.

Results

Carotenoid plasma level, immune responses and physical condition

The experimental manipulation of diet results in significant effects in birds' plasma carotenoid levels, with treatment being significant (P=0.041) as well as the interaction between time and treatment (P=0.036) (Table 1). Considering separately the two time periods, there was no initial difference in plasma carotenoid levels ($F_{1,11}$=1.097, P=0.320), but there was a significant difference after the treatment (control males 1.20±0.446 μg/ml; β-supplemented males 7.22±2.192 μg/ml; $F_{1,11}$=7.253, P=0.023) (Fig. 2). For the PHA-P response, there was a significant effect of time (P=0.023) and a significant interaction between time and treatment (P=0.003) (Table 1). Analysing the treatment at each time separately, there was no initial difference in the response to PHA-P challenge ($F_{1,11}$=2.847, P=0.122), but β-supplemented males showed a higher response to PHA-P challenge than control males ($F_{1,11}$=8.949, P=0.014) after the treatment (Fig. 3). Ectoparasite load changed significantly with time (before treatment 12.06±10.10; after treatment 0.08±0.29 P<0.0001). There were no effects of the experimental manipulation on SRBC and physical condition (Table 1).

Table 1 Repeated measures ANOVA for an effect of diet supplementation on plasma carotenoid levels, immune challenges (PHA-P and SRBC responses), physical condition and ectoparasite load

		Time	Treatment	Time × Treat.
Plasma carotenoid levels	*F*	1.02	5.48	5.84
	P	0.337	0.041	0.036
PHA-P response	*F*	7.86	1.80	18.29
	P	0.023	0.216	0.003
SRBC	*F*	3.13	2.07	0.86
	P	0.115	0.188	0.381
Physical condition	*F*	1.97	1.92	0.41
	P	0.191	0.196	0.537
Ectoparasite load	*F*	34.98	0.01	0.03
	P	<0.0001	0.908	0.861

Model included time (before/after treatment) and treatment as factors. Values are *F* values (*F*) and probabilities (*P*)

Carotenoid-based colouration

SWS in plumage colouration varied significantly with time ($P<0.0001$), and there was an interaction between time and treatment ($P=0.009$) (Table 2). Considering separately the two time periods, there was no initial difference in colour expression between the two groups of males (SWS ratio: $F_{1,17}=0.578$, $P=0.458$). However, after the food supplementation experiment, the plumage saturation of β-supplemented males was higher than that of the control males (SWS ratio $F_{1,17}=6.942$, $P=0.018$) (Fig. 4). No effects were found on DC or the size of the yellow patch, both in the forehead and chest (Table 2).

Mate choice experiment

The two treatment groups of males were submitted to a female choice test in the following breeding season in order to assess the effect of colour change in mate choice. Females clearly preferred β-supplemented males, spending significantly more time facing them (Wald $\chi^2=9.952$; $df=1$; $p=0.002$) (Fig. 5) than control males. This was not affected by the activity of males (Wald $\chi^2=0.687$; $df=1$; $p=0.407$).

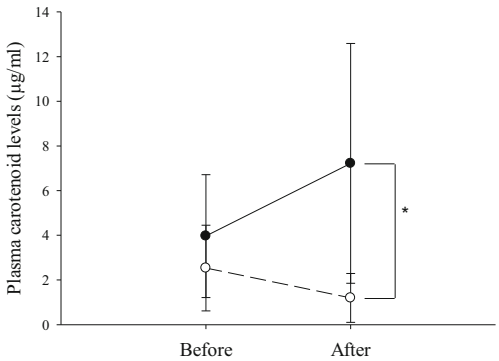

Fig. 2 Plasma carotenoid levels (mean±standard deviation) of β-supplemented males (*solid symbol*) and control males (*open symbol*) before and after the feeding treatment. *$P<0.05$

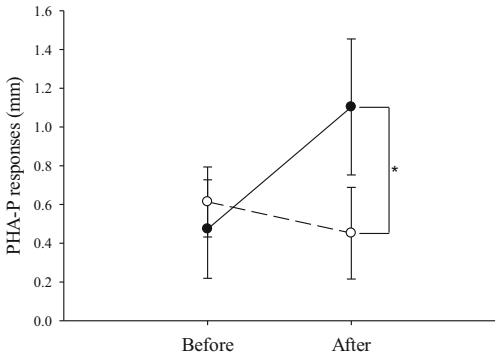

Fig. 3 PHA-P responses (mean±standard deviation) of β-supplemented males (*solid symbol*) and control males (*open symbol*) before and after the feeding treatment. *$P<0.05$

Discussion

Our experiments revealed that by increasing β-carotene availability in the diet of male serins, we also observed an increase in their carotenoid plasma concentration and in their immune response. Our treatment also affected plumage ornament expression and attractiveness to females, since males given extra β-carotene became more colourful and were preferred by females over control males. Our three initial predictions were confirmed, which supports an indirect role for carotenoid colouration as signal of condition. Serins undergo a single post-reproductive moult, which takes place long before the signal is relevant for sexual display. So, carotenoid-based plumage colouration in this species should, most likely, predict long-term aspects of individual's quality.

Table 2 Repeated measures ANOVA for an effect of diet supplementation on plumage colouration, SWS, double cone and size of forehead and chest patch

		Time	Treatment	Time × Treatment
SWS	*F*	98.12	1.982	8.739
	P	<0.0001	0.178	0.009
Double cone	*F*	0.127	0.833	1.080
	P	0.726	0.375	0.314
Forehead patch	*F*	3.136	1.038	0.004
	P	0.098	0.326	0.949
Chest patch	*F*	1.106	0.072	0.215
	P	0.309	0.792	0.649

Model included time (before/after treatment) and treatment as factors. Values are *F* values (*F*) and probabilities (*P*)

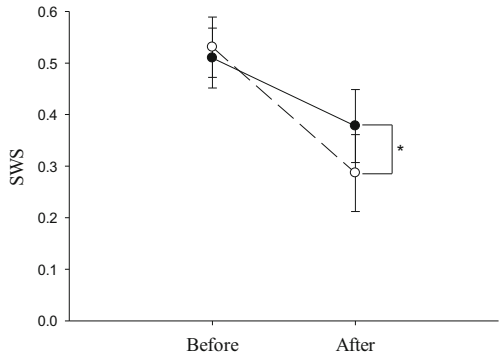

Fig. 4 SWS (mean±standard deviation) of β-supplemented males (*solid symbol*) and control males (*open symbol*) before and after the feeding treatment. After manipulation, there was a significant difference between treatments. *$P<0.05$

Carotenoid plasma level, immune responses and physical condition

First, we wanted to determine if the addition of β-carotene to the diet of males affected their immune response. Indeed, β-supplemented males showed an increase in plasma carotenoid concentration and a stronger immune response. Other experiments showed that an enhanced diet promoted a higher plasma lutein circulation on great tits *Parus major* (Peters et al. 2011) and a nutritional deprivation diminished plasma carotenoids in male goldfinches (McGraw et al. 2005). β-Carotene is a carotenoid particularly linked to an antioxidant role and is an immunoenhancer (Bendich 1989; Chew 1993), and it also serves as vitamin A precursor, which is involved in several basic metabolic processes, including growth, development, vision, immune system and reproduction (D'Ambrosio et al. 2011).

Our results revealed that the β-carotene supplementation affected particularly the cellular immunity since the immune response of males was only significant in the PHA-P test, which measures the immunity mediated by cells, involving both innate and adaptive responses of the immune system (Martin et al. 2006; Tella et al. 2008). No differences were found between supplemented and control males in the SRBC test, which measures T-dependent humoral immunity. In male red grouse *Lagopus lagopus scoticus*, comb colour and condition predicted the PHA-P response (Mougeot 2008), and in red-legged partridges, *Alectoris rufa* colouration, plasma carotenoids and cell-mediated immune response were positively correlated (Pérez-Rodriguez et al. 2008). Also, in a diet experiment during moult of great tits, carotenoid supplementation increased PHA-P response, but not SRBC response (Peters et al. 2011). And, in a recent meta-analysis, Simons et al. (2012) found that PHA was the only measure of immune function that was associated with carotenoid levels. The immune challenge response seems to be species-specific, however, as contrasting results can be found in different species. Navara and Hill (2003) reported no differences in immune responses to increasing doses of carotenoid supplementation in American goldfinches. But, in another study, carotenoid supplementations increased both cell-mediated and humoral immune responses in zebra finches (McGraw and Ardia 2003). While in some other studies, β-carotene was responsible for the increase in antibody titres in cockerels (McWhinney and Bailey 1989) and wild gulls (Blount et al. 2001). One possible mechanism for the action of β-carotene on immune stimulation could be through mitochondrial function on the immune system. Mitochondria have well-known roles in cellular metabolism, generating energy for physiological processes, regulating stress responses and signalling for apoptotic cell death (West et al. 2011; Galluzzi et al. 2012). Besides, mitochondria could have a central role in the innate immunity (West et al. 2011). As several mitochondrial functions are regulated by vitamin A (Stillwell and Nahmias 1983), an indirect action of β-carotene and vitamin A on immune system could occur.

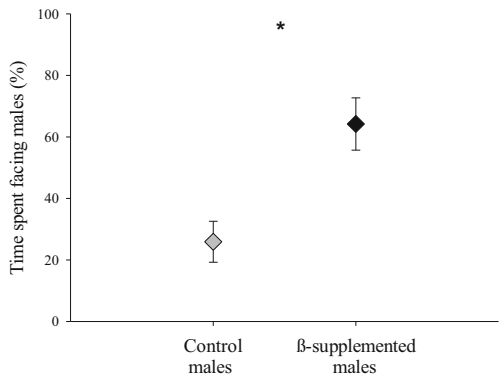

Fig. 5 Time spent by females in association with β-supplemented males and control males (mean±standard deviation, $N=12$), in the following spring. *$P<0.05$

Although there was an increase in immune response of the β-supplemented male serins, we found no differences in physical condition or in ectoparasite load between these and control males. Ectoparasite load after moult was actually very similar between the two treatment groups and close to zero, which is probably due to moulting occurring in the nearly aseptic aviary environment.

Carotenoid-based colouration as a signal

We also wanted to determine if supplementation with β-carotene could affect the yellow colour expression of males, which could only occur through an indirect effect, as β-carotene is not in the metabolic pathway to produce the pigments to be deposited in the birds' feathers (McGraw 2006).

Under natural conditions, birds have a limited access to carotenoids due to environmental constrains or experience. A critical assumption for the evolution of sexually selected signals is that they have to be honest about the traits that they

signal, which depends on them being costly (Grafen 1990; Searcy and Nowicki 2005). A trade-off between using carotenoids for immune defence and for colour signalling was suggested as being the main cause for the maintenance of the honesty of the signal (Blount 2004). Similarly, carotenoid colouration was associated with antioxidant function (Chew and Park 2004). However, this trade-off hypothesis was questioned in the sense that, most likely, carotenoids are not environmentally limiting. Instead, it was proposed that carotenoid colouration was not advertising their direct antioxidant function but was acting as an indirect indicator of uncoloured resources including antioxidant molecules which could protect carotenoids from oxidation (Hartley and Kennedy 2004). In accordance with this hypothesis, effects of vitamins and uncoloured carotenoids were observed on the colouration of fishes (Pike et al. 2007), reptiles (Kopena et al. 2014) and birds (Bertrand et al. 2006b). Bertrand et al. (2006b) found an additive effect of carotenoids and melatonin, which is a free radical scavenger in bill colour in zebra finches. If the concentration of carotenoids in feathers is not directly related to their availability for this and other purposes, but is instead a signal of general health of individuals, then improving the individuals' condition, e.g. by making other carotenoids available, should also affect the expression of the signal. By supplementing male serins with β-carotene, we assessed the indirect effects that high concentration of carotenoid may have as antioxidants, as immune enhancers, or as acting in other metabolic processes which ultimately affect the transformation of lutein and zeaxanthin into canary xanthophylls A and B that are mobilised into feathers. We did not provide any lutein to the birds, and this resulted in a decrease in plumage SWS colouration in both treatments. But, the decrease was much less accentuated in the β-carotene-supplemented males, being the difference significant. Thus, β-carotene partially prevented the decrease in carotenoids in the plumage. Our results agree with carotenoid colouration being an indirect indicator of condition-associated resources which can also be carotenoid-dependent and susceptible of improvement by carotenoid availability in serins.

More recently, a vitamin A-redox hypothesis was proposed by Hill and Johnson (2012) linking carotenoid colouration and individual oxidative state and immune function, through the cellular pathways that are regulated by vitamin A, which is an essential micronutrient and plays a major role in several basic life processes, as redox homeostasis. The hypothesis was advanced to explain the signalling role of red colouration in birds, since carotenoids can act as vitamin A precursors and also be deposited in feathers after modification. In yellow-coloured birds, the process is different since the main carotenoids in feathers, canary xanthophylls A and B, are obtained from oxidation of lutein and zeaxanthin, while pro-vitamin A carotenoids follow a different pathway and are either transported to the liver or cleaved into retinal (Debier and Larondelle 2005; von Lintig 2010). The authors consider that their hypothesis can also apply to species with yellow colouration, albeit in a different way, since vitamin A not only regulates carotenoid uptake and transport, but also acts as antioxidant maintaining redox levels. Although we do not know by which mechanism β-carotene is affecting these birds' colouration, our results are in accordance with this hypothesis, since the increase of the main vitamin A precursor availability had an effect in the colour saturation of males. The vitamin A-redox hypothesis establishes a detailed set of possible connections between carotenoid colouration and the biochemical and molecular processes of vitamin A homeostasis and oxidative state. One possibility to explain our results is that the ingestion of β-carotene could affect the trait through the role of vitamin A in mediating oxidative state which then would affect carotenoid ornamentation. Another possibility is that besides its role as vitamin A precursor, β-carotene could have a role on the immune system or on individual homeostasis, thus affecting immune responses and plumage colouration. Finer testing on the mechanisms that relate condition with signal expression in species with carotenoid colouration is needed. There are only a few examples linking non-pigmentary substances and colouration of a carotenoid-based sexual trait. Beak colour was associated with carotenoid and vitamin A concentration in spotless starlings (*Sturnus unicolor*) (Navarro et al. 2010), and a non-pigmentary antioxidant enhanced bill colour in zebra finches (Bertrand et al. 2006b).

Female choice for more colourful males

Since carotenoid-supplemented males had more saturated colouration, we expected that females would prefer them. As predicted, females spent significantly more time in association with β-supplemented males than control males, indicating a preference for more colourful males. The behaviour of males had no influence on female choice. This is in accordance with a few previous studies performed in species with carotenoid-based plumage colouration (Hill 1990, 1994; Johnson et al. 1993; Sundberg 1995; reviewed in Hill 2006; Leitão et al. 2014). Mate choice based on carotenoid ornaments could provide both direct and indirect benefits to females. It makes evolutionary sense if colouration signals good genes or healthy males. Good gene models propose that females gain indirect benefits by choosing males by an indicator of quality (Evans et al. 2004), improving their offspring fitness. Females can also have direct benefits, through parental care. Another benefit with both a direct and indirect component is that they could choose to mate with less parasitised males (Figuerola et al. 2003) or which are healthier. In our study, females would benefit by choosing more colourful males which had a higher immune condition.

In conclusion, our results support the hypotheses that carotenoid-based ornamentation is an honest sexual signal, encoding information about pigment access, nutritional condition and health. These results support an indirect signalling role for yellow carotenoid colouration, in accordance with previous suggestions (Hartley and Kennedy 2004). They also agree with the vitamin A redox hypothesis, through an indirect way (Hill and Johnson 2012). Further work should try to understand the mechanisms than maintain this association.

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Ethical standards All experiments were performed in accordance to Portuguese legislation for research on animal behaviour and were conducted under license permits: 258/2009/CAPT to PGM and 259/2009/CAPT to ST, by Instituto da Conservação da Natureza e da Biodiversidade (ICNB).

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