BEHAVIORAL ECOLOGY - ORIGINAL RESEARCH

Female blue tits with brighter yellow chests transfer more carotenoids to their eggs after an immune challenge

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Abstract Female ornaments are present in many species, and it is more and more accepted that sexual or social selection may lead to their evolution. By contrast, the information conveyed by female ornaments is less well understood. Here, we investigated the links between female ornaments and maternal effects. In birds, an important maternal effect is the transmission of resources, such as carotenoids, into egg yolk. Carotenoids are pigments with antioxidant and immunomodulatory properties that are crucial for females and developing offspring. In blue tits, we evaluated whether ultraviolet (UV)/blue and yellow feather colouration signals a female's capacity to allocate carotenoids to egg yolk. Because mounting an immune response is costly and trade-offs are more detectable under harsh conditions, we challenged the immune system of females before laying and examined the carotenoid level of their eggs afterward. A positive association between feather carotenoid chroma and egg carotenoid level would be expected if yellow colouration signals basal immunity. Alternatively, if female colouration more generally reflects maternal capacity to invest in reproduction under challenging conditions, then other components of colouration (i.e. yellow brightness and UV/blue colouration) could be linked to maternal capacity to invest in eggs. No association between egg carotenoid levels and UV/blue crown colouration or female yellow chest chroma was found; the latter result suggests that yellow colouration does not signal immune capacity at laying in this species. By contrast, we found that, among females that mounted a detectable response to the vaccine, those with brighter yellow chests transmitted more carotenoids into their eggs. This result suggests yellow brightness signals maternal capacity to invest in reproduction under challenging conditions, and that male blue tits may benefit directly from choosing brighter yellow females.

Keywords Plumage colouration · Female ornaments · Immune challenge · Maternal effects · Sexual selection

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Introduction

In many species, conspicuous traits are present both in males and females (Kraaijeveld et al. 2007; Clutton-Brock 2009). These traits in females have long been considered non-functional byproducts of sexual selection on male traits, resulting from a genetic correlation between male and female traits (Lande 1980). Alternatively, it may be that sexual and/or social selection is acting on both male and female traits (Kraaijeveld et al. 2007; Clutton-Brock 2009; Tobias et al. 2012). Like male ornaments, female ornaments could signal reproductive or survival benefits and be involved in male mate choice (Amundsen 2000; Clutton-Brock 2009; Edward and Chapman 2011). Male mate choice has received theoretical support (Johnstone

et al. 1996; Kokko and Johnstone 2002; Servedio and Lande 2006; Hooper and Miller 2008) and has been documented and demonstrated in various taxa: fishes (Amundsen and Forsgren 2001), insects (Byrne and Rice 2006), reptiles (Lebas and Marshall 2000), mammals (Domb and Pagel 2001), and birds (Amundsen et al. 1997; Griggio et al. 2005; Torres and Velando 2005). Male mate choice is particularly expected to occur in species with biparental care, but some studies have shown that it can also occur in species with no parental care. For instance, male differential allocation attributable to female phenotype has been shown in the fowl species, *Gallus gallus* (Cornwallis and O'Connor 2009).

Males may receive direct or indirect benefits (good genes) by mating with more ornamented females. In order to understand the nature of these benefits, it is essential to determine the information that is signalled by female ornaments. Previous correlative studies have shown both positive and negative relationships between female ornaments and proxies of female quality, such as female body condition, age, immunocompetence, survival, feeding rate, or fecundity (e.g. reviewed in Amundsen 2000 and see Simmons and Emlen 2008; Gasparini et al. 2009; Hanssen et al. 2009; Lehikoinen et al. 2009; Weiss et al. 2009; Boulet et al. 2010; Gladbach et al. 2010; Huchard et al. 2010; Baldauf et al. 2011 for recent results in birds, mammals, reptiles, fishes, and insects). To date, experiments are rarer but more consistently report links between female ornaments and maternal quality (e.g. Roulin et al. 2000, 2001; Blount et al. 2002; McGraw et al. 2005; Doutrelant et al. 2008; Gasparini et al. 2009; Kekäläinen et al. 2010; Martinez-Padilla et al. 2011; but see Smiseth and Amundsen 2000).

An essential component of female quality for males is the maternal capacity to invest in progeny (Blount 2004). Prenatal maternal effects (Mousseau and Fox 1998) are non-genetic mechanisms by which females can affect offspring phenotypes and fitness. In egg-laying species, they can involve the transmission of important components into eggs, e.g. immunoglobulins, hormones, or carotenoids (Bortolotti et al. 2003; Groothuis et al. 2005; Boulinier and Staszewski 2008).

Carotenoids present in egg yolks play a central role during embryo development and at hatching. They are immunomodulators that regulate and stimulate the immune system (i.e. the production of lymphocytes and the phagocytic ability of neutrophils and macrophages: Lozano 1994; Olson and Owens 1998; Møller et al. 2000; Krinsky 2001). There are positive relationships between carotenoid levels in yolks (or carotenoid availability during laying) and offspring health, survival, growth, and fledging success (e.g. Blount et al. 2002; Biard et al. 2005; McGraw et al. 2005; Ewen et al. 2009). Indeed, embryo and nestling

development are associated with elevated oxidative stress because of rapid growth. Thus, maternal carotenoids are particularly important for detoxification at this stage (Surai et al. 1999). In addition, because of their role in the regulation and activation of the immune system, maternal carotenoids can have a positive effect on nestling responses to parasites. For instance, higher levels of carotenoids in eggs enhance the offspring immune response (Saino et al. 2003; Biard et al. 2007), and nestlings hatched from eggs with higher levels of carotenoids are better able to compensate for the negative effects of ectoparasitism (Ewen et al. 2009).

Carotenoids are also important for adult health. To date, most experimental studies using carotenoid supplementation found that carotenoid-supplemented individuals have a stronger immune response than controls (e.g. Surai 2002; Blount et al. 2003; Hasselquist and Nilsson 2012 for some reviews and McGraw and Ardia 2003; McGraw et al. 2011; Peluc et al. 2012). Carotenoids can only be acquired from food and are often considered a limited resource subject to trade-offs. This is especially true during egg laying, when female metabolism is high (e.g. Nilsson and Raberg 2001). For instance, in blue tits (*Cyanistes caeruleus*), carotenoid supplementation during egg laying resulted in a significant increase in the carotenoid concentration in egg yolks (Biard et al. 2005).

The trade-off between carotenoid allocation to eggs and self-maintenance is predicted to be modulated by female characteristics and immune activity. For instance, Navara et al. (2006) and Williamson et al. (2006) found a positive relationship between female condition and carotenoid transfer, and Saino et al. (2002a) showed that female immune status influences carotenoid concentration in eggs, with vaccinated females transmitting fewer carotenoids. In addition, because carotenoids are of crucial importance for nestlings and maternal condition, female ornaments might be predicted to signal a female's capacity to transfer carotenoids into eggs to potential mates, either because highly ornamented females have higher basal immunity or because they are in better condition and can thus invest more in offspring.

To date, only a handful of correlative studies have investigated the link between female ornaments and egg carotenoid content. Because certain types of integument colouration rapidly mirrors changes in condition (Faivre et al. 2003; Velando et al. 2006), a strong association is expected between female ornaments and carotenoid content because ornament production, immunity, and reproduction occur at the same period. The two experimental studies that have investigated this topic support this prediction; they found positive relationships between female integument colouration and the level of beneficial components in eggs following carotenoid supplementation (in

black-backed gulls, *Larus fuscus*, Blount et al. 2002; in zebra finches, *Taeniopygia guttata*, McGraw et al. 2005). For ornaments that are produced during the non-breeding season, predictions are more complex as these ornaments are linked to conditions occurring long before egg production. Correlative studies that investigated the links between such ornaments and egg carotenoid content provided mixed results: there was no correlation between feather yellow colouration and egg carotenoid level in blue and great tits (Biard et al. 2005; Szigeti et al. 2007; Remes et al. 2011), but a positive correlation was found between egg carotenoid level and the immaculateness of the white cheek patch in great tits (Remes et al. 2011).

We used a handicapping approach to investigate whether the yellow and UV/blue colouration of feathers indicates a female's capacity to allocate carotenoids in blue tits. We challenge the immune system of females before laying and examined the carotenoid level of their eggs afterward.

We predicted:

1. A stronger relationship between ornament colouration and egg carotenoid content in the experimental group than in the control group. Trade-offs, as well as links between traits and estimates of individual quality, are more detectable when environmental conditions are harsher (Van Noordwijk and de Jong 1986; Doutrelant et al. 2008; Morales et al. 2008). As a consequence, the importance of carotenoids is clearer when adults are faced with immune challenges as opposed to when they are experiencing normal conditions (Costantini and Møller 2008).
2. A link between feather carotenoid chroma and egg carotenoid content if carotenoid-based colouration signals immune capacity. Feathers with more carotenoids have higher chroma (Isaksson et al. 2008b). So, if females that are able to deploy carotenoids to their plumage at moult have high basal immunity, we would expect the yellow chroma of the plumage to be positively associated with egg carotenoid content.
3. A link between egg carotenoid content and other components of yellow or UV/blue colouration if, alternatively, female colouration generally reflects maternal capacity to invest in reproduction under challenging conditions. Mounting an immune response is costly (Lochmiller and Deerenberg 2000; Bonneaud et al. 2003). As a result, some components of colouration reflecting female condition or female capacity to invest in reproduction could be linked to maternal capacity to invest in eggs, independently of the carotenoid-based trade-off between immunity and reproductive investment in signalling and offspring. A previous experimental study in this species suggested

that yellow brightness is an important colour parameter that indicates maternal capacity to invest in reproduction under challenging conditions: females experimentally forced to produce a replacement clutch produced more eggs and recruited more offspring when brighter in colour (Doutrelant et al. 2008).

Materials and methods

Model system and study population

Blue tits have a slightly dimorphic structural UV/blue crown colouration, but monomorphic yellow carotenoid-based chest colouration (Andersson et al. 1998; Hunt et al. 1998; Doutrelant et al. 2008). The UV/blue crown appears to be involved in mutual mate choice (Andersson et al. 1998; Hunt et al. 1999). It affects maternal decisions and allocation to nestlings (Sheldon et al. 1999; Delhey et al. 2003; Griffith et al. 2003; Limbourg et al. 2004; Johnsen et al. 2005; Kingma et al. 2009 but see Dreiss et al. 2006) as well as intraspecific competition in both sexes (Alonso-Alvarez et al. 2004; Rémy et al. 2010; Vedder et al. 2010; Midamegbe et al. 2011). By contrast, to date, yellow colouration (mostly brightness) seems more linked to parental quality (Senar et al. 2002; Doutrelant et al. 2008).

The blue tit population we studied is located in Montarnaud, in the south of France (43°40′N, 03°40′E), and occurs in a broadleaved deciduous forest of downy oaks (*Quercus pubescens*). The population has been followed since 1991 and breeds in Schwegler B1 nest boxes. Each year, breeding birds are captured and their reproduction is monitored (Blondel et al. 2006). The average clutch size in our population is around ten (Doutrelant et al. 2008). No egg dumping has ever been observed, and 14 % of offspring across 46 % of the nests are the product of extra-pair copulations (Charmantier and Blondel 2003).

Bird capture and treatment

Using mist nets, we captured 45 females near their nest boxes prior to egg laying (10–20 March 2008). Females were randomly assigned to one of two groups: vaccinated or control. Twice as many females were included in the vaccinated group as in the control to ensure that the final sample size included enough females that had both laid eggs and mounted a measurable immune response. Indeed, the stimulation of an immune response might prevent egg-laying because it is physiologically costly and/or vaccination might not work in some individuals (Hamers et al. 2002).

We vaccinated female blue tits against the Newcastle disease virus (NDV). NDV is an avian virus that has been

previously used in avian studies (e.g. Saino et al. 2002b; Staszewski et al. 2007; Staszewski and Siitari 2010; Garnier et al. 2012). It is frequently found in domestic birds but has an extremely low prevalence in natural bird populations (Camenisch et al. 2008). For instance, the World Organisation for Animal Health (OIE) reports that no infected birds were found among the 3,049 wild birds tested in Switzerland. Vaccinated females received subcutaneous injections of 10 μL of NDV, an inactivated vaccine (Nobivac Paramixo; Intervet, France) ($n_{vaccinated\ females} = 30$). Control females were injected with an identical volume of phosphate-buffered saline (PBS) ($n_{control\ females} = 15$).

At the time of a bird's capture, its tarsus length was measured with a digital calliper to the nearest 0.02 mm, and its body mass was measured to the nearest 0.5 g using a Pesola spring balance. In addition, six blue feathers from the crown and eight yellow feathers from the chest were collected for colour measurements (see below). Bird sex and age (1 year old versus >1 year old) were determined using the colour of the wing coverts (Svensson 1992). Before their release, females were ringed with a uniquely numbered metal ring from the French Museum of Natural History (Centre de Recherches par le Baguage des Populations d'Oiseaux) that was used to identify them at the end of the laying period; the identity of previously ringed females was noted.

Nest monitoring and egg collection

Nest boxes were inspected every 4 days starting on 20 March 2008. Once the first egg had been laid in the population, nests were inspected every 2 days, and eggs were marked in order to know their order in the laying sequence (±1 day) within the clutch (blue tits lay one egg per day). One day after the clutch size stopped increasing, and just after the start of incubation, we recaptured the breeding females; we identified them by ring number and determined their experimental group (control or vaccinated). All their eggs were then collected. A total of 22 of the 30 vaccinated females and 12 of the 15 control females had been recaptured at the end of the egg-laying period. Females injected with NDV (vaccinated) or PBS (control) had similar probabilities of initiating egg laying (respectively 0.80 and 0.73, Pearson's χ^2-test: $\chi^2 = 0.24$, $P = 0.62$).

Verifying vaccination response

It has been demonstrated that if a female has antibodies circulating in her blood, she will systematically transfer them to her eggs (Grindstaff et al. 2003; Grindstaff 2008).

To evaluate if all vaccinated females responded to vaccination, we analysed NDV levels in egg yolks [see Electronic supplementary material (ESM) online]. We detected anti-NDV antibodies in the eggs of 13 out of the 22 vaccinated females. There was no difference in age, tarsus length, condition at treatment, or blue or yellow colouration for females that transferred anti-NDV antibodies versus those that did not (all $P > 0.10$). They also had the same return rate ($P = 0.40$).

The absence of anti-NDV antibodies in some vaccinated females may be due to several reasons, such as genetic background, poor somatic condition, or improperly implemented vaccination (Hamers et al. 2002; Aguilera and Amat 2007; Staszewski et al. 2007; Krams et al. 2012). As a consequence, some of the nine females that did not transfer anti-NDV antibodies to their eggs may or may not have paid the cost of mounting an immune response. As our aim was to compare females that unambiguously paid the cost of mounting an immune response to the controls, we excluded this heterogeneous group of nine non-responsive females from our statistical analyses. Whether these nine females were included or not did not change the results: the same variables were retained during model selection. Our experimental group was thus redefined to comprise only the 13 responsive females, and we use the term "vaccinated females" to refer to them solely from this point forward.

Vaccinated females and control females had similar laying dates ($F_{1,23} = 0.06$, $P = 0.81$), clutch sizes ($F_{1,22} < 10^{-3}$, $P = 1$), plumage colouration (yellow brightness, $F_{1,23} = 2.72$, $P = 0.11$; yellow chroma, $F_{1,23} = 1.63$, $P = 0.21$; blue brightness, $F_{1,23} = 0.24$, $P = 0.63$; blue hue, $F_{1,23} = 3.37$, $P = 0.08$), tarsus length ($F_{1,23} = 0.67$, $P = 0.42$), and body mass at the time of treatment ($F_{1,22} = 0.50$, $P = 0.48$). The number of days between the treatment (vaccination or PBS) and the first egg laid varied between 14 and 33 days (mean ± SD = 24.08 ± 5.57 days) and was similar for control and vaccinated females ($F_{1,22} = 1.27$, $P = 0.27$).

Determination of carotenoid levels in egg yolks

Yolk carotenoid concentrations were determined using colourimetry (Strand 1998). For carotenoid extraction, 50–70 mg of the egg yolk was mixed with a corresponding volume of acetone (1 μL of acetone for 0.1 mg of yolk). Samples were kept overnight at −20 °C and then centrifuged at 13,000g at 4 °C for 10 min. We determined the optical density (OD) at 450 nm of 125 μL of the supernatant using a microplate photometer (Multiskan Ascent; Therma Oy, Finland). Lutein is the main carotenoid in blue tit eggs (Biard et al. 2005; Isaksson et al. 2008a). We therefore used a serial dilution of a commercial solution of standard lutein (xanthophyll Sigma X-6250) to generate a

standard curve and determine the relationship between the OD value and the carotenoid concentration in the egg yolks. The carotenoid concentration is expressed in micrograms per gram of egg yolk.

We analysed the carotenoid content of 45 eggs that tested positive for anti-NDV antibodies (from one to seven eggs per vaccinated female). We analysed 47 eggs from the 12 control females (from three to six eggs per control female). The egg order in the laying sequence of the vaccinated versus control females was similar within their respective clutches ($F_{1,23} = 3.30$, $P = 0.08$, mean egg order in the laying sequence $= 7.5$, range $= 1$–13 for vaccinated females; mean egg order in the laying sequence $= 6.3$, range $= 1$ to 13 for control females). Order in the laying sequence of eggs and the time delay between treatment and laying did not affect carotenoid content (respectively, $F_{1,66} = 0.003$, $P = 0.96$, $F_{1,65} = 0.47$, $P = 0.50$). The number of days the eggs were incubated had a significant effect on carotenoid content ($F_{1,23} = 8.41$, $P = 0.01$).

Differences in yolk carotenoids were significantly greater among clutches than within clutches ($F_{24,67} = 8.70$, $P < 10^{-3}$), a result previously reported in blue tits and other species (e.g. Szigeti et al. 2007; Isaksson et al. 2008a; Holveck et al. 2012).

Colour measurement and colour variables

Feather colouration was measured following the same procedure as in Doutrelant et al. (2008, 2012). Feather reflectance was measured with an AVASPEC-2048 spectrometer (Avantes, the Netherlands); a deuterium-halogen light source (AVALIGHT-DH-S lamp; Avantes) that covers the 300- to 700-nm spectral range visible to blue tits (Hart et al. 2000); and a 200-μm fibre optic probe. Colour spectra information was extracted using Avicol software version 3 (Gomez 2009). For both colour patches (UV/blue and yellow), brightness was calculated as the mean reflectance over the 300- to 700-nm range (defined as the area under the curve divided by the width of the interval 300–700 nm) so as to include the entire range of colour to which birds are sensitive. Chroma and hue were calculated based on the shape of the reflectance spectra (e.g. Andersson et al. 1998; Delhey et al. 2003). For UV/blue crown colouration, UV chroma was the average of the reflectance between 300 and 400 nm divided by the mean reflectance over the 300- to 700-nm range. Hue was the wavelength at maximum reflectance. For yellow chest colouration, chroma was the difference between the maximal reflectance between 500 and 700 nm and the reflectance at 450 nm divided by the average reflectance $[R_{max(500–700\ nm)} - R_{450}]/R_{AV}$. This measure is strongly linked to the level of carotenoids incorporated in feathers because carotenoids maximally absorb around 450 nm (Andersson and Prager 2006; Isaksson et al. 2008b).

We did not calculate hue parameters for yellow colouration because of the double-peaked nature of these carotenoid-based spectra. Our measurements were significantly repeatable with all $P < 0.0001$ (Lessells and Boag 1987) (UV/blue crown brightness repeatability $R = 0.65$ and hue $R = 0.75$; yellow chest brightness $R = 0.44$ and chroma $R = 0.68$).

Statistical analyses

We investigated the link between female colouration and egg carotenoid levels in control females (injected with PBS) and vaccinated females (measurably responded to NDV vaccine). The dependant variable was yolk carotenoid concentration, which was log transformed to normalize the distribution. As several eggs from the same clutch were included in the analyses, we always included the identity of the female as a random variable. The explanatory variables were treatment (vaccination versus control), female plumage colouration (UV/blue crown—brightness, hue, and UV chroma; yellow chest—brightness and chroma), and female characteristics that may affect female carotenoid allocation: female body condition, female tarsus length, and female age (two classes: 1 year old versus >1 year old). Female body condition was estimated by examining the effect of body mass (measured the day of vaccination) using a type III model in which tarsus length was one of the explanatory variables (Garcia-Berthou 2001; Green 2001). In addition, we included the interactions between female plumage colouration and treatment. We also included as explanatory covariables the laying date of the first egg, clutch size residuals (derived from the regression of clutch size on laying date), and the stage of incubation. All correlations between explanatory variables or covariables were low and thus unlikely to lead to collinearity problems ($-0.53 <$ all $\rho < 0.36$, all P from 0.9 to <0.001; Belsley et al. 2004).

Data were analysed in R 2.8.1 (Crawley 2005) using linear mixed models (LMMs). We began with full models and then employed backward selection procedures with type III errors to obtain the most parsimonious model containing only significant effects ($P \leq 0.05$). Because of the high number of variables included in the models, we also used a forward selection procedure in which we first tested the effect of all the variables and interactions separately and then included the variables and interactions with a $P \leq 0.05$. As the results were similar, we present those found with the backward procedure.

Results

We found a significant interaction between the experimental treatment (vaccinated or control) and female yellow

chest brightness on the carotenoid levels of egg yolks (Table 1). This indicates that although vaccinated females allocated fewer carotenoids to their eggs than control females (Table 1; estimate $\pm$ SE: -1.31 ± 0.31), this carotenoid allocation was different for bright and dull yellow females in both treatment groups.

When running the model separately for vaccinated and control females (and including all the significant factors listed in Table 1), it appears that yolk carotenoid concentration significantly increased with yellow chest brightness in vaccinated females (estimate $\pm$ SE $= 0.04 \pm 0.01$, $F_{1,5} = 7.88$, $P = 0.038$; Fig. 1), whereas yellow brightness was not correlated with yolk carotenoid concentration in control females (estimate $\pm$ SE -0.04 ± 0.02, $F_{1,5} = 5.48$, $P = 0.066$, Fig. 1).

The results of our analysis also show that higher levels of yolk carotenoids were found in females that were older, had longer tarsi, laid later, laid smaller clutches, and were in poorer condition at the time of treatment (Table 1; Figs. S2, S3, S4, S5, S6 in the ESM). No effects of UV/blue crown hue and brightness or yellow chest chroma were found (all $P > 0.13$).

Discussion

The aim of our study was to experimentally test whether female colouration signals maternal capacity to invest in reproduction. We used a handicapping experiment in which we challenged the immune system of female birds, and then related carotenoid content in their eggs to their feather colouration. If feather carotenoid chroma were linked to female basal immunity, we predicted an association between this colour trait and egg carotenoid content. However, no association was found. Alternatively, if female colouration reflects maternal condition and/or maternal capacity to invest in reproduction, we expected
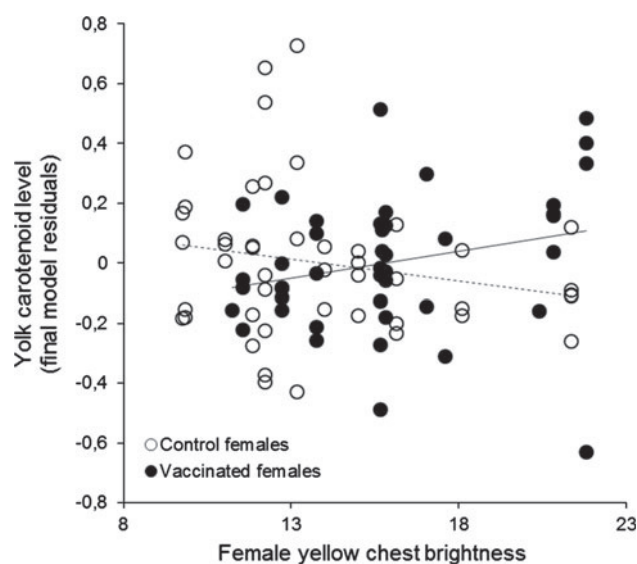

Fig. 1 Relationship between egg carotenoid concentration and female blue tit yellow chest brightness in control and vaccinated females. Statistics conducted in each group (see "Results" section) showed that yolk carotenoid concentration significantly increased with female yellow brightness in immune-challenged females but not in control females (linear regression lines are shown)

other components of colouration to be linked to egg carotenoid content. We found an interaction between vaccination against NDV and female yellow brightness. Immunochallenged females transferred fewer carotenoids to their eggs than did control females, and brighter yellow females transmitted more carotenoids to their eggs than duller females in the vaccine-treated but not in the control group. This result suggests that, under challenging conditions, brighter females are able to invest more in reproduction than dull ones.

In tits and other bird species, yellow feather chroma reflects carotenoid deposition (e.g. Saks et al. 2003a; Andersson and Prager 2006; Shawkey et al. 2006; Isaksson et al. 2008b; Peters et al. 2008) and is condition dependent

Table 1 Results from linear mixed models for carotenoid transfer

Yolk carotenoid levels	Estimate	SE	df	F	P
Treatment (vaccinated < control)	-1.31	0.31	1, 15	0.02	0.001
Female yellow chest brightness	-0.03	0.01	1, 15	0.05	0.021
Female body mass at time of treatment	-0.18	0.05	1, 15	5.96	0.046
Female tarsus length	0.59	0.08	1, 15	35.88	$<10^{-3}$
Female age (adult > yearling)	-0.22	0.10	1, 15	8.96	0.040
Egg laying date	0.02	0.007	1, 15	20.84	0.005
Clutch size residuals	-0.09	0.02	1, 15	24.65	0.002
Treatment × female yellow brightness	0.09	0.02	1, 15	20.63	$<10^{-3}$

Parameter estimates $\pm$ SE, df, F values, and P values are only indicated for variables included in the final model with $P \leq 0.05$. $n = 91$ eggs laid by $n = 24$ females: 44 eggs from 12 vaccinated females (one female was excluded due to a missing body mass value) and 47 eggs from 12 control females

(blue tits, Doutrelant et al. 2012; other species, e.g. McGraw et al. 2001; Hill et al. 2004; Shawkey et al. 2006; Peters et al. 2008). If females able to deploy more carotenoids to their plumage at moult have high basal immunity, we would predict that they would allocate more carotenoids to their eggs following an immune challenge. However, given the fact that yellow feather chroma at moult does not relate to egg carotenoids suggests that this colour parameter is not a reliable signal of female immune capacity at laying, maybe because immunity is highly seasonal (Hawley and Altizer 2011).

The positive relationship between yellow chest brightness and egg carotenoid content under challenging conditions may be interpreted in two ways. Our prediction was that if female colouration generally reflects maternal capacity to invest in reproduction under challenging conditions, then components of colouration other than yellow chroma could be linked to the maternal capacity to invest in eggs. Under this hypothesis, the positive link between yellow brightness and egg carotenoid level in immuno-challenged females would indicate that brighter yellow females are better-quality mothers who can invest more in reproduction. However, because pigment-based colours are subtractive (i.e. more deposited pigment absorbs more light, Andersson and Prager 2006), individuals that are brighter yellow are sometimes considered to contain fewer carotenoids in their feathers (Andersson and Prager 2006). An alternative hypothesis is that, if brighter females have fewer carotenoids in their plumage, brighter yellow females are females of lower quality with lower survival prospects who consequently invest more in current reproduction when faced with disease (i.e. terminal investment hypothesis, Clutton-Brock 1984; Bonneaud et al. 2004; Reaney and Knell 2010; Bowers et al. 2012).

At this stage, although it is not possible to fully disentangle these two explanations, we have more arguments in favour of the hypothesis that brightness reflects maternal capacity to invest in reproduction under challenging conditions. First, in disagreement with the terminal investment hypothesis, we found that within vaccinated females, brighter yellow females had a significantly higher local survival rate (i.e. return rate) than duller ones (general linear models, estimate $\pm$ SE $= -0.28 \pm 0.11$, $P = 0.01$). Second, egg carotenoid content is positively linked to two out of three recognised proxies of quality—age and tarsus length. Last, most studies that have investigated the link between feather carotenoid content and brightness did not find a negative relationship (Saks et al. 2003a; Shawkey et al. 2006; Isaksson et al. 2008b; Hill et al. 2009), and it has been suggested that yellow brightness may partly result from feather structure (Shawkey and Hill 2005) and melanin deposition in feathers (Isaksson et al. 2008b). Thus,

yellow chest brightness might signal something else besides feather carotenoid content, which could explain why in blue tits (Senar et al. 2002; Doutrelant et al. 2008 but see García-Navas et al. 2012) and other species (Saks et al. 2003b; Reudink et al. 2009), it is yellow brightness and not carotenoid chroma that appear to signal parental investment. So, most elements are in favour of the hypothesis that brighter females are higher-quality mothers, but clearly more studies are needed to clarify the physiological mechanisms linking the yellow brightness of plumage to the maternal transfer of carotenoids.

Variability in carotenoid transfer capacities, and possibly the ability to mount an immune response, has been related to several factors such as genetic differences, stress level, androgen level, and behavioural differences (Boulinier and Staszewski 2008; Owen et al. 2010; Ardia et al. 2011; Krams et al. 2012). It might consequently be interesting to examine the relationships between brightness and these factors. In particular, it would be highly interesting to study hormonal and behavioural differences, as previous results have shown that brighter yellow females are less aggressive in our population (Midamegbe et al. 2011). Such work would enhance our understanding of the mechanisms underlying the positive relationships found, but would not change the conclusion that yellow brightness is linked to proxies of parental investment in blue tits (Senar et al. 2002; Doutrelant et al. 2008; this study) and other species (Saks et al. 2003b; Reudink et al. 2009), and thus may be a signal of parental investment.

In the control group, brighter yellow females tended to allocate fewer carotenoids to their eggs. However, this result was not significant and confirmed results obtained in other years or study populations (Biard et al. 2005; Szigeti et al. 2007; Holveck et al. 2012) where no relationship between yellow colouration and egg carotenoid concentration was found.

We found two results suggesting that carotenoids may be limiting in our populations. Females laying bigger clutches had lower carotenoid concentrations in their eggs, and females laying later transferred more carotenoids to their eggs (see Szigeti et al. 2007 for identical results). The concurrent increase in yolk carotenoids with laying date can be explained by the seasonal increase in the availability of carotenoid-rich food, such as leaf-eating Lepidopteran caterpillars (Partali et al. 1987; Isaksson and Andersson 2007). Indeed, Tummeleht et al. (2006) found that great tit plasma carotenoid levels increased seasonally and in tandem with caterpillar availability. This hypothesis of carotenoid limitation fits with the results obtained by Biard et al. (2005) in blue tits and by other studies in another species (e.g. Blount 2004). However, it should be noted that the egg carotenoid levels in our blue tit study

population (30.96 ± 13.76 μg/g egg yolk, range 11.84–75.44) are higher than those reported for another blue tit population (Biard et al. 2005, 21 ± 1.6 μg/g, range 9.0–56.9) and great tits in Sweden (Isaksson et al. 2008a, about 15 μg/g), but are lower than those in great tits in Estonia (Hõrak et al. 2002, about 48 μg/g). Obviously, additional work will be required to determine whether or not carotenoids are limiting in our unchallenged study population.

We did not find a significant link between UV/blue crown colouration and yolk carotenoids. In Doutrelant et al. (2008), UV/blue crown colouration was positively linked to female survival but not female reproductive success. In addition, Midamegbe et al. (2011) found that UV/blue crown colouration can be used as a badge-of-status in the context of female-female competition. It is thus likely that female UV/blue colouration signals another aspect of female quality, such that yellow chest colouration and UV/blue crown colouration would be complementary signal traits. In many species, it is common for different traits to signal different components of quality (Candolin 2003; Roulin 2009; Bro-Jorgensen 2010; Gomez et al. 2011).

In conclusion, our results suggest that carotenoid-based colouration does not signal female immune capacity during reproduction and that plumage colouration indicates maternal capacity to invest in reproduction under adverse reproductive conditions. Such signals could directly benefit males and be sexually selected. Experiments testing male mate choice are now needed to validate this hypothesis. Moreover, experiments designed to determine the physiological mechanisms explaining the observed links between yellow brightness and maternal propensity to invest in reproduction under challenging conditions are needed. More generally, our results promote the current re-evaluation (Kraaijeveld et al. 2007; Clutton-Brock 2009) of the role of sexual selection in the evolution of female ornaments. They suggest that maternal effects are important elements to consider when investigating the signalling content of female ornaments.

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