doi: 10.1111/j.1420-9101.2006.01203.x

Carotenoid and protein supplementation have differential effects on pheasant ornamentation and immunity

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Keywords:

body condition;
immune capacity;
secondary sexual characters;
supplemental feeding.

Abstract

A currently popular hypothesis states that the expression of carotenoid-dependent sexual ornaments and immune function may be correlated because both traits are positively affected by carotenoids. However, such a correlation may arise for another reason: it is well known that immune function is dependent on nutritional condition. A recent study has suggested that the expression of ornaments may too depend on nutritional condition, as males in good nutritional condition are better at assimilating and/or modulating carotenoids. Thus, carotenoid-dependent ornaments and immune function may be correlated because both are dependent on nutritional condition. To elucidate if, and how, ornamentation and immune function are linked, pheasant diets were supplemented with carotenoid and/or protein in a fully factorial experiment. Carotenoid treatment affected wattle coloration and tail growth, but not cellular or humoral immunity. Immunity was unrelated to males' initial ornamentation including wattle colour. Males in better body condition, measured as residual mass, increased their wattle coloration more when carotenoid supplemented. Protein positively affected humoral but not cellular immunity, but had no effect on ornaments. Cellular, but not humoral, immunity increased with male body condition. Thus, there was no evidence that an immune-stimulatory effect of carotenoids resulted in wattle coloration honestly signalling immune function, but wattle coloration may still signal male body condition.

Introduction

Females prefer to mate with ornamented males in many species. This preference may have evolved because ornament expression signals male phenotypic quality so that females mating with more ornamented males obtain direct or indirect (genetic) benefits (Andersson, 1994). In many birds, sexual ornaments are coloured by the inclusion of carotenoids (Badyaev & Hill, 2000), and males with redder and more saturated ornaments attract more females (Hill, 1990; Zuk *et al.*, 1995) or dominate other males (Ligon *et al.*, 1990; Pryke & Andersson, 2003). Also the growth of ornaments such as spurs and feather plumes may be positively affected by carotenoids. This is because keratinocyte proliferation is sensitive to oxidative metabolites and free radicals (von Schantz *et al.*, 1999) whereas carotenoids may function as antioxidants and protect against such oxidative stress (Krinsky, 2001; Surai, 2002). Although it is often stated that carotenoid-dependent ornaments signal male quality, there is no consensus concerning the mechanism by which this signalling is achieved. Instead, several mechanisms have been proposed whereby carotenoid-dependent ornaments could reliably signal male quality (Olson & Owens, 1998).

A currently popular hypothesis suggests that carotenoid-dependent ornaments signal the bearer's health status (Lozano, 1994; von Schantz *et al.*, 1999). This idea is based on the finding that carotenoids are important for immune function; carotenoids have been shown to both enhance immune responsiveness (Bendich & Shapiro,

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1986; Fenoglio *et al.*, 2002; Chew & Park, 2004) and protect against free radicals produced by the immune system (von Schantz *et al.*, 1999). It has therefore been argued that there is a trade-off between allocating carotenoids to ornaments and immune defence, and that only healthy males can afford the immunological cost of investing carotenoids in ornaments (von Schantz *et al.*, 1999; Alonso-Alvarez *et al.*, 2004). If so, carotenoid-dependent ornaments could honestly signal the health status of males. By basing their mate choice on such ornaments, females, on average, will mate with healthier males, which might give direct and, if immune function is heritable, indirect benefits. This hypothesis is often interpreted as predicting a positive correlation between ornamentation and immune function (von Schantz *et al.*, 1999; Faivre *et al.*, 2003; Peters *et al.*, 2004), an interpretation that is based on the assumption that there is more variation among males in health status than in how carotenoids are allocated between immune function and ornaments (cf. van Noordwijk & De Jong, 1986). However, we suggest that such a positive correlation between carotenoid dependent ornaments and immune function may also arise in another, indirect way: Hill (2000) recently showed that expression of carotenoid dependent ornaments is dependent on nutritional condition independently of the access to carotenoids. Furthermore, it is also well known that immune function is dependent on nutritional condition. For example, different amounts of food (Saino *et al.*, 1997; Klasing, 1998) or dietary protein content (Lochmiller *et al.*, 1993; Kidd, 2004) have been shown to affect immune responsiveness and/or the ability to handle infections. If both carotenoid-dependent ornamentation and immune function are dependent on availability of nutrients, these traits may be correlated even if there is no direct link between carotenoids and immune function. Under this scenario, females mating with more ornamented males will on average mate with males with a better nutritional condition and may thus gain direct or, if nutritional condition has a partly genetic component, indirect benefits.

To date, empirical tests of the idea that carotenoid-dependent ornaments reveal the health status of its bearer because of a carotenoid-based trade-off between sexual ornamentation and immune function have demonstrated conflicting results regarding the role of carotenoids. Correlational studies have demonstrated positive (Saks *et al.*, 2003), no (Navara & Hill, 2003) or negative (Faivre *et al.*, 2003) associations between strength of immune responses and ornamentation. A better way to test the trade-off hypothesis is through experimental manipulation: if ornaments honestly reveal the immunological capacity of males because males have to trade carotenoids between these two functions (Lozano, 1994; von Schantz *et al.*, 1999), dietary supplementation with carotenoids should increase both ornament expression and immune function. However, although two studies have found a parallel positive effect of carotenoid supplementation on strength of immune responses and ornamentation in zebra finches (*Taeniopygia guttata*) (Blount *et al.*, 2003; McGraw & Ardia, 2003), a study on house finches (*Carpodacus mexicanus*) found no such relationship (Navara & Hill, 2003). Reasons for this variation may be that carotenoids are not always limiting in birds, which normally have much higher plasma carotenoid levels than for example mammals (Navara & Hill, 2003) or that the complex relationship between carotenoids and immunity means that a higher level of carotenoids is not always positive (Chew & Park, 2004). Clearly, more studies of the connection between immune defence and carotenoid dependent ornaments are desirable. In particular, it would be important to investigate if any relationship between ornament expression and immunity is directly caused by carotenoids, or if it acts through indirect effects on nutritional condition (Hill, 2002).

The ring-necked pheasant (*Phasianus colchicus*) is a sexually dimorphic species, with males being larger and extensively ornamented compared with the cryptic females. The males' various ornaments are used in both intra- and inter-sexual selection (reviewed in Mateos, 1998). Pheasants have a socially polygynous mating system and males do not participate in the rearing of young, but females receive direct benefit from males in the form of guarding during feeding (Ridley & Hill, 1987). In a Swedish population of pheasants, among measured traits (age, weight, spur length, tarsus length, tail length, bill length and wing length, but not wattle size or coloration), spur length was the most important trait for female mate choice, with a female preference for long-spurred individuals (von Schantz *et al.*, 1989; Göransson *et al.*, 1990). In a study of pheasants in Spain, the length of male spurs was correlated to body condition [in second year (SY) males] and dominance [in after second year (ASY) males]; however, there was no female preference for long-spurred males (Mateos & Carranza, 1996). Instead, female choice seemed to be based on characteristics of the wattle as well as the length of ear tufts and tail feathers (Mateos & Carranza, 1995). In a study on English pheasants, the brightness of the wattle was important for female choice (Hillgarth & Wingfield, 1997). The red wattle in male pheasants is a striking character that can be engorged and is used during courtship as well as in dominance behaviour between males (Taber, 1949; Mateos, 1998; Papeschi *et al.*, 2003). Parasite-free males demonstrated a higher degree of wattle inflation and were more successful in attracting harems (Hoodless *et al.*, 2002). Little information exists concerning what affects pheasant wattle colour, but wattles and combs, in general, owe their colour to blood in the richly vascularized superficial dermis and presumed carotenoid pigments in the epidermis (Stettenheim, 2000). Dietary variation in carotenoids affects grouse comb colour (Egeland *et al.*, 1993) and pheasant wattle colour (Ohlsson *et al.*, 2003).

The aim of this study was to investigate if any relationship between pheasant sexual ornaments and immunity could be caused by a direct effect of carotenoids on both immune function and ornament expression or, alternatively, by both immunity and ornament expression being sensitive to males' nutritional condition. In a factorial experiment we therefore tested if two putative ornaments, viz. tail length and comb colour, and two aspects of the immune system, viz. cell-mediated (Erf, 2004) and humoral (Scott, 2004) immunity, were affected by dietary supplementation of carotenoid and protein. If there is a carotenoid-based trade-off between ornaments and immune function, carotenoid supplementation should enhance both traits. In contrast, if immune function and ornamentation are linked because both are dependent on nutritional condition, we expect that protein supplementation should enhance both traits. As male body condition can be affected by other factors than the ability to find and assimilate nutrients, we also related immunity and ornamentation to nonexperimental variation in male body condition.

Methods

Experimental design

First-year male pheasants were bought from a local breeder and housed in an outdoor aviary. When the experiment was initiated, the aviary was divided into four 40 m^2 pens. Pheasants were randomly assigned to one of four experimental groups, each confined to one of these four pens. During the experiments, pheasants were moved between pens so that each group was housed sequentially in all four pens. Water and food were available *ad libitum* throughout the study.

The experiment consisted of a 2×2 factorial design in which pheasants received food with low or high protein content and drinking water with or without canthaxanthin. When the experiment started on 12 April 2002, there were 15, 16, 17 and 16 males in the groups assigned to the low/low (carotenoid/protein), low/high, high/low and high/high treatment respectively. The initial tarsus length ($F_{3,57} = 0.18$, $P = 0.91$), mass ($F_{3,57} = 0.39$, $P = 0.76$), body condition (measured as residual mass when corrected for tarsus length; $F_{3,56} = 0.95$, $P = 0.42$), wattle colour score ($F_{3,58} = 0.16$, $P = 0.92$), cellular immune response (see below) ($F_{3,60} = 1.17$, $P = 0.33$) and primary humoral immune response (see below) ($F_{3,57} = 0.50$, $P = 0.69$) did not differ between experimental groups.

As food we used Allfoder veg® with a low (8%) and Allfoder start® with a high (24%) protein content (Skånska Lantmännen, Malmö, Sweden). The two types of food do not differ in their content of other components. The recommended protein content in the food for adult pheasants range from 10% to 20% (Woodard *et al.*, 1977; Sheppard *et al.*, 1998). In addition to grain, with a protein content of *c.* 10%, pheasants in spring utilize food with a higher protein content such as green shoots (15–25%) and insects (40–77%) (Sheppard *et al.*, 1998; Draycott, 2002). Thus our treatment was selected to contain less or more protein than their natural diet. Drinking water was or was not supplemented with 200 ppm canthaxanthin (Rosche®). We selected canthaxanthin because it is commercially available, is readily taken up by poultry (Koutsos *et al.*, 2003b), is naturally ingested or synthesized from other carotenoids by birds (Surai, 2002), is the dominating carotenoid in the skin of wild pheasants (30% of total carotenoids, Czeczuga, 1979), suppresses oxidative stress (Palozza & Krinsky, 1992; Surai *et al.*, 2003) and affects immunity in poultry (El-Hafeez *et al.*, 2000; see also Koutsos *et al.*, 2003a). Differential feeding was continued for 60 days until the experiment was ended.

Measurements

Morphological measurements were taken 30 days before and 59 days after differential feeding started. All birds were weighed to the nearest 0.1 g on a digital balance (Mettler Toledo®, Greifensee, Switzerland) and had their tarsus length measured to the nearest 0.01 mm using digital callipers. Body condition was calculated as the residual mass when correcting for tarsus length in a linear regression (Merilä *et al.*, 2001; Schulte-Hostedde *et al.*, 2005). In addition, potential ornaments were measured. Spur length was measured from the tip to the distal edge of the tarsus. From this measurement we then subtracted the thickness of the tarsus to obtain a measure of the actual length of the spur. The length of the longest tail-feather was measured with a ruler. On the first occasion, most tail feathers were worn and could therefore not be measured. On this occasion, we plucked the two longest tail feathers. At the end of the experiment the length of the longest regrown feather was measured. If the feather was broken, the measurement was discarded.

We measured wattle colour with a colorimeter (Colortron II, Light Source Inc., San Rafael, CA, USA). We took two measurements on each of a male's two wattles and calculated the mean hue, saturation and brightness using the four measurements at each occasion. Because the three colour measurements were strongly correlated, an overall colour score was calculated for each male as the first principal component of these (Hill, 1998). This score was strongly correlated with the hue, saturation and brightness of the wattle ($r > 0.84$ in all cases except for one correlation with brightness in which $r = 0.52$). We always measured colour at the same spot on the wattle (above the eye). All measurements were taken by the same person (T.O.). Measurements were taken 30 days before the experiment started, and 45 and 59 days after differential feeding started. A male with a higher score had a redder, more saturated and darker wattle.

Immunology

To estimate cellular immune responsiveness (i.e. immunity mediated by cells like macrophages, natural killer cells and cytotoxic T-lymphocytes) we injected 1 mg phytohemagglutinin (PHA) (Cat. L-8754; Sigma, St Louis, MO, USA) dissolved in 0.1 mL sterile phosphate-buffered saline (PBS) into the patagium ('wing-web') and measured the subsequent swelling as an assay of *in vivo* T-cell-mediated immune responsiveness (Goto *et al.*, 1978). Following the recommendations of Smits *et al.* (1999), we did not inject PBS to measure the swelling as a control in the other wing. We measured the thickness of the wing-web in the right wing to the nearest 0.01 mm with a micrometer immediately before and 24 h after injection. Measurements were taken as follows: one person held the bird, down and feathers were plucked or turned aside from the swollen area, and the micrometer was tightened just until the skin began to twist. The median of three measurements was used. All measurements were taken by the same person (M.G.). In all analyses, we have used the size of the swelling caused by the PHA-injection after subtracting the starting value. The wing-web test was performed twice, 5 days before the differential feeding started and 59 days after differential feeding started.

We measured humoral immune responsiveness (i.e. immunity mediated by antibodies) as the antibody production in response to two nonpathogenic antigens. All birds were first vaccinated 30 days before the dietary manipulation started and then again 52 days after differential feeding started. The antibody responses to these vaccinations are referred to as primary and secondary responses respectively. On each occasion birds were immunized with 250 μL of a vaccine containing diphtheria and tetanus toxoid (SBL, Stockholm, Sweden) intramuscularly in the pectoral muscle. Hence, we obtained two measures of humoral immune responsiveness. Just before vaccination and 13–14 (primary response) or 7 (secondary response) days after vaccination, blood samples (*c.* 300 μL, taken from the wing vein) were collected in tubes with heparin and stored on ice until centrifugation (856 g for 7 min) later the same day. The plasma was then extracted and stored at −20 °C until later analysis. Antibody titres were analysed using an enzyme-linked immunosorbent assay (see Ohlsson *et al.*, 2003).

Both the primary and secondary responses against the two different antigens were strongly correlated (primary, $r_{59} = 0.57$, $P < 0.0001$; secondary, $r_{57} = 0.59$, $P < 0.0001$). We therefore created a principal component for the primary and secondary responses separately. These new variables correlated strongly with the diphtheria and tetanus responses ($r > 0.88$ in all cases).

Statistical analyses

Sample size differs between analyses for several reasons. One pheasant died during the experiment. Two pheasants lost their rings and although the identity of their experimental group was clear, initial and final measurements could not be matched. Some measures of spur and tail length were discarded, because it was noted in the field protocols that they were broken. In a few cases, measurements were missed; in particular final mass was not measured for the high carotenoid high protein group. One very deviant measure of mass was discarded because we suspected it to be erroneous (but inclusion did not qualitatively change results).

Statistical analyses were performed using paired *t*-tests, Pearson correlations, general linear models or mixed models (SAS 9.1 PROCS T-TEST, CORR, GLM and MIXED respectively). When testing the effect of the experiment, we used the measurement before differential feeding started as covariate when applicable. When controlling for the effect of the primary response, the background level (i.e. before vaccination) of antibodies against tetanus and diphtheria did not significantly explain the strength of the secondary response (partial correlation, diphtheria, $r_{52} = -0.19$, $P = 0.17$; tetanus, $r_{52} = -0.01$, $P = 0.94$) and was therefore not used as an additional covariate in the analyses. Because of missing measurements (see above) we could not test for interaction effects of experimental treatments on final mass and body condition. Separate analyses of the low carotenoid and low protein group gave qualitatively the same results, but only the two-way analyses are presented for simplicity. We checked for normality and homoscedasticity. The antibody titres and tail lengths were log-transformed to normalize data. Mixed models were used in cases in which we included more than one measurement from the same individual in the analyses, for example wattle colour score in relation to experimental treatment or when analysing immune responses in relation to body condition of birds. In these models, pheasant identity was used as a random factor and time was included as a fixed factor when it explained a significant proportion of the variation. We allowed for heterogeneous variance among the two time periods if this improved the AIC of the model (Littell *et al.*, 1996). We used the Satterthwaite approximation of denominator degrees of freedom (Littell *et al.*, 1996).

Results

Mass and body condition

There was a marginally significant positive effect of carotenoid treatment on body mass [LS means 1130.4 ± 18.0 (SE) g and 1180.7 ± 32.3 g for the low and high protein group], but no effect of protein treatment (Table 1). The body condition of males on the high-carotenoid-diet increased more than that of males on the low carotenoid diet (−13.51 ± 9.20 and 40.04 ± 14.95 g for the low- and high-carotenoid diet males respectively; Table 1) and protein treatment had a marginally

Table 1 The effect of dietary supplementation of protein and canthaxanthin (carotenoid) on various measures of male pheasants. Tested with mixed (wattle colour) or general linear models.

		Carotenoid		Protein		Interaction		Initial measure	
Variable	Ddf	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Mass	38	3.95	0.054	0.39	0.53	*	*	94.93	<0.0001
Condition	37	9.43	0.0040	2.85	0.10			46.59	<0.0001
Wattle colour	57.6†	27.73	<0.0001	0.08	0.77	0.07	0.80	5.77	0.020
Tail length	41	12.37	0.0011	0.88	0.35	0.07	0.80	n.a.	
Cellular immune response	57	0.40	0.53	1.09	0.30	0.60	0.44	0.05	0.82
Humoral immune response	54	0.99	0.33	5.68	0.021	0.05	0.82	24.60	<0.0001

Ddf, denominator degrees of freedom; n.a., not available.
*Interaction not tested (see *Methods*).
†57.2 for initial measure.

significant effect (−7.12 ± 12.3 g vs. 49.18 ± 23.0 g for the low- and high-protein group; Table 1).

Ornament development

Both post-experimental measures of wattle colour were strongly correlated within individuals ($r_{60} = 0.75$, $P < 0.0001$) and not significantly different (paired-$t_{61} = 0.2$, $P = 0.83$). Wattle coloration was significantly affected by the carotenoid treatment, but not by the protein treatment (Table 1; Fig. 1). Colour was affected by interaction between carotenoid treatment and body condition (mixed model including effects of treatment, body condition and initial colour score, the effect of the interaction $F_{1,79} = 9.87$, $P < 0.01$) because there was a positive relationship between colour score and body condition in the high-carotenoid treatment group ($b = 0.0084$, $F_{1,29} = 18.57$, $P < 0.001$) but not in the low-carotenoid treatment group ($F_{1,49} = 0.56$, $P = 0.46$). Thus, either birds in high body condition were better able to assimilate carotenoids or birds assimilating more carotenoids gained more in body condition (Fig. 2).

Males on a diet supplemented with carotenoids grew longer tail feathers than those on a low-carotenoid diet (Fig. 3; Table 1), but there was no effect of protein treatment. Tail regrowth was unrelated to body

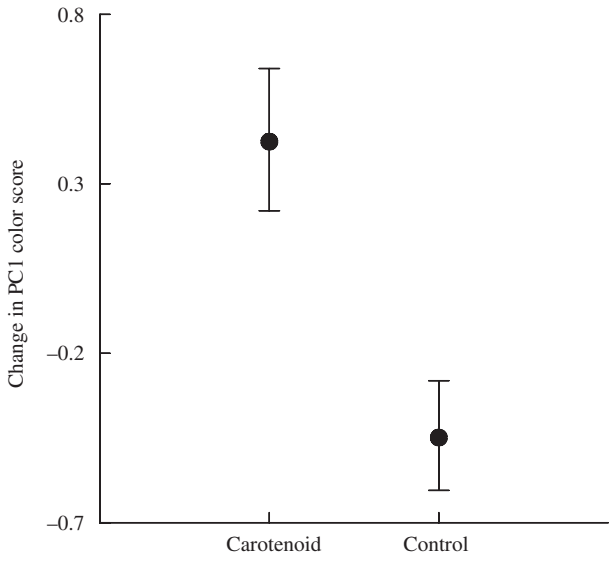

Fig. 1 Change in wattle colour in relation to carotenoid treatment. The colour score was calculated as the first principal component for hue, saturation and brightness of the wattle; the mean of the second and third measurements at the end of the treatment period subtracted by the initial colour score is used here. Data for both protein treatments included. SE indicated. For statistics, see Table 1.

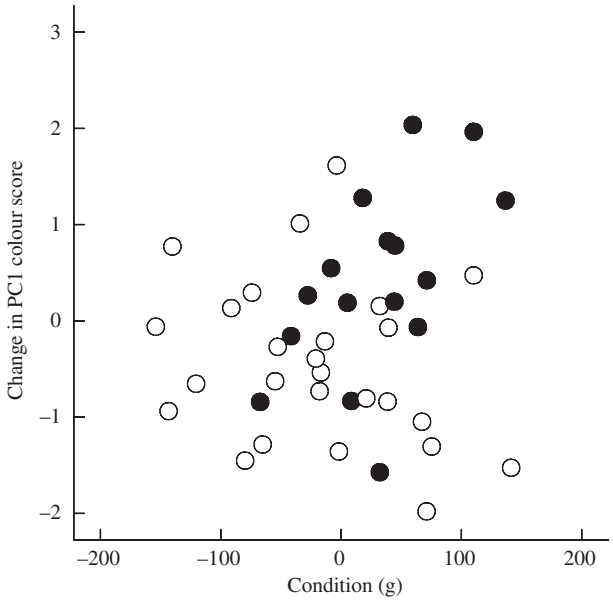

Fig. 2 Change in wattle colour (see Fig. 1) in relation to the condition of male pheasants measured as residual mass. The colour score is the mean of the second and third measurement at the end of the treatment period subtracted by the initial colour score before the experiment. Open dots = low carotenoid diet, filled dots = high carotenoid diet. Data for both protein treatments included. For statistics, see text.

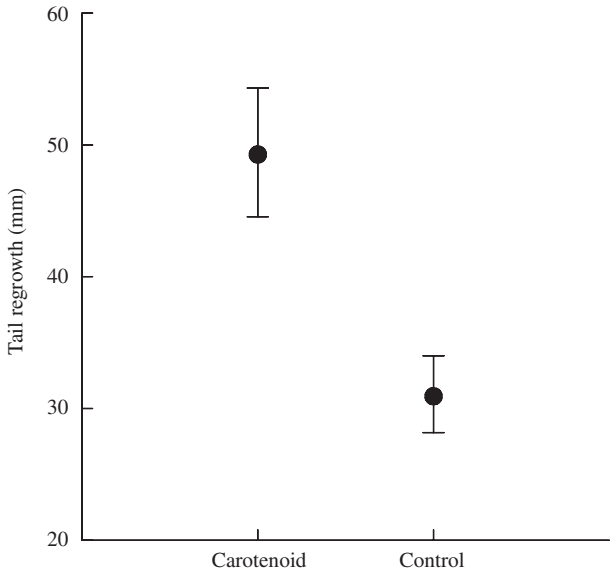

Fig. 3 Regrowth of a central tail feather in relation to carotenoid treatment. Data for both protein treatments included. SE indicated. For statistics, see Table 1.

condition (general linear model including carotenoid treatment, the effect of body condition $F_{1,28} = 0.1$, $P = 0.75$).

The analysis of spur length was hampered by extensive wear on the spurs. For example, whereas most males demonstrated some growth of the spurs, 32% of spur measurements were more than 1 mm shorter at the second measurement, presumably because of spur wear or spur breakage. Change in spur length was affected by an interaction between the treatments ($F_{1,59} = 4.13$, $P = 0.047$). However, this result was not upheld, if reductions in spur length larger than 1 mm were assumed to be caused by spur breakage ($F_{1,55} = 0.01$, $P = 0.91$; for males with one presumably broken spur the measurement of the alternate spur was used). Thus, as average spur growth was small (mean = 0.61 mm, SD = 0.74, $n = 56$), spur breakage had a large influence on calculations. Spur length was unrelated to body condition ($P > 0.1$ whether potentially broken spurs are excluded or not).

Immunological response

There was no relationship between the first (pre-experiment) and second (post-experiment) measure of the cell-mediated immune response ($r_{60} = -0.07$, $P = 0.60$). The strength of the response was somewhat stronger for the second time period (2.24 + 0.59) compared with the first (1.97 + 0.73; paired-$t_{61} = 2.26$, $P < 0.05$). The cell-mediated response was not affected by experimental treatment (Table 1). The response was also unrelated to the colour score of the wattle (repeated measures ANOVA using measurements both before and after the experimental treatment; effect of colour score: $F_{1,76.5} = 1.64$, $P = 0.20$). The relationship between wattle colour and the strength of the cell-mediated immune response did not differ between treatment groups (effect of interaction between treatment group and immune response on wattle colour, $F_{1,58} = 0.01$, $P = 0.94$). The strength of the immune response tended to be related to the length of the spur (repeated measures ANOVA using measurements both before and after experimental treatment; effect of spur length: $F_{1,80.2} = 3.94$, $P = 0.051$). However, there tended to be an interaction between time and the effect of spur length ($F_{1,83.8} = 2.83$, $P = 0.097$) because the effect was significant before the experiment ($r_{60} = 0.29$, $P < 0.05$) but not by the end of the experiment ($r_{60} = 0.08$, $P = 0.54$). The strength of the second response was not related to the growth of the spurs ($r_{58} = 0.22$, $P = 0.089$). This result was unaffected by excluding potentially broken spurs (see above; $r_{54} = 0.23$, $P = 0.090$). The strength of the second response was also unrelated to the length of the regrown tail feather ($F_{1,43} = 0.28$, $P = 0.60$).

The strength of the immune response to PHA was related to the body condition of the birds, but not significantly so (repeated-measures ANOVA using measurements both before and after the experimental treatment, the effect of body condition: $F_{1,90.5} = 3.74$, $P = 0.056$; Fig. 4) and this effect did not differ between the first and second PHA-injection (interaction between body condition and time, $F_{1,97.6} = 0.03$, $P = 0.86$).

The primary and secondary humoral immune responses were significantly associated ($r_{57} = 0.54$, $P < 0.0001$) and the secondary responses were stronger both for tetanus (paired-$t_{58} = 7.29$, $P < 0.0001$) and diphtheria (paired-$t_{58} = 8.04$, $P < 0.0001$). The secondary response was unrelated to the carotenoid treatment, but significantly affected by protein supplementation (Fig. 5; Table 1).

The secondary response was unrelated to the expression of ornamental traits (mean wattle colour, $F_{1,58} = 0.03$, $P = 0.87$; spur length; $F_{1,58} = 0.05$, $P = 0.82$; length of re-grown tail feather; $F_{1,42} = 0.12$, $P = 0.73$). This also held true when controlling for the effect of the primary response ($P > 0.3$ in all cases). Similarly, there was no relationship between the strength of the humoral immune response and wattle colour ($F_{1,105} = 0.44$, $P = 0.51$) or spur length ($F_{1,108} = 0.69$, $P = 0.41$) in repeated measures analyses including both pre- and post-treatment measurements. The relationship between wattle colour and the strength of the secondary response did not differ between treatment groups (effect of interaction between treatment group and immune response on wattle colour, $F_{1,56} = 0.77$, $P = 0.39$). The secondary response was unrelated to the body condition of the bird ($F_{1,39} = 0.70$, $P = 0.41$) and this result was the same when statistically controlling for the primary response ($F_{1,37} = 0.24$, $P = 0.62$). Similarly, the response was

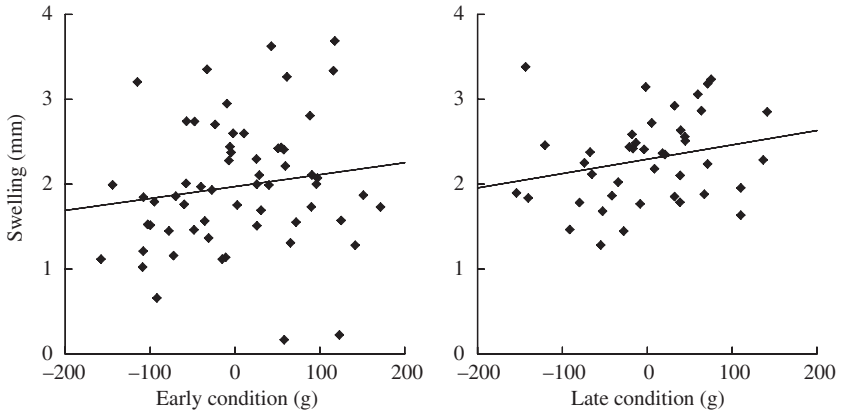

Fig. 4 The strength of the immune response after an injection with phytohemagglutinin before and after the experimental treatment. For statistics see text.

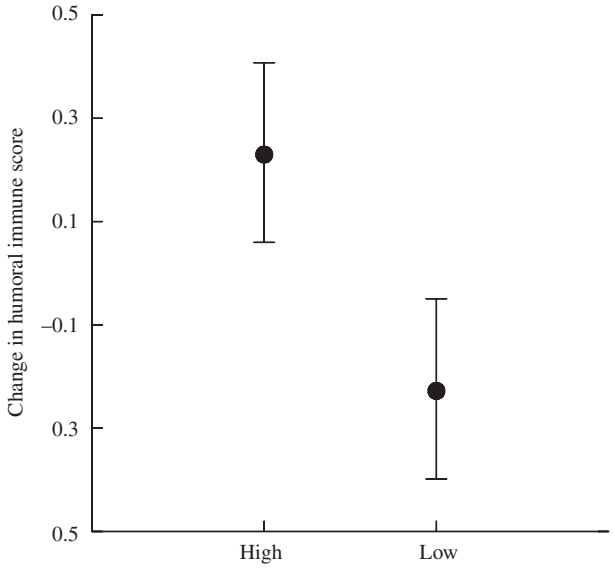

Fig. 5 Change in the strength of the humoral immune response in relation to whether the diet was low or high in protein content. Humoral immune score is the principal component for the titres of antibodies against tetanus and diphtheria. Data for both carotenoid treatments included. SE indicated. For statistics, see Table 1.

unrelated to body condition in a repeated measures analysis ($F_{1,94.8} = 0.03$, $P = 0.86$).

The strength of the cellular and humoral immune responses were not related to each other, either before ($r_{59} = 0.23$, $P = 0.074$) or after ($r_{58} = 0.019$, $P = 0.89$) the treatment period.

Discussion

It has been suggested that the positive effect of carotenoids on both immune function and ornament expression causes ornaments to honestly reveal the health status of males because males have to trade carotenoids between these two functions (Lozano, 1994; Von Schantz *et al.*, 1999). However, in contrast to some recent studies (Blount *et al.*, 2003; McGraw & Ardia, 2003), but in agreement with others (Fenoglio *et al.*, 2002; Navara & Hill, 2003), our study did not support this idea. Although dietary supplementation with a carotenoid affected the colour of the wattle (see also Ohlsson *et al.*, 2003) and tail regrowth, it did not affect immune function, and we found no relationship between immunological competence and ornament expression. There could still be a link between ornament expression and immunity if both ornament expression and immunity are affected by general nutrition. In agreement with several other studies (Lochmiller *et al.*, 1993; Klasing, 1998), we found that immunity was affected by the nutritional content of the males' food. However, in contrast to two other studies (Hill, 2000; McGraw *et al.*, 2005), we did not find that the nutritional value of the adult diet affected ornament expression. Thus, in contrast to some previous studies, our results do not lend any clear support to either a carotenoid-based trade-off between ornamentation and immune defence or the idea that ornaments and immunocompetence are correlated because both are dependent on nutritional status. Below we discuss possible reasons behind this variation in results.

Variation in the effect of carotenoids

Carotenoids have been suggested to affect immunity because they, and their derivates, function as antioxidants (Lozano, 1994; von Schantz *et al.*, 1999). This may result in positive effects on both cellular and humoral immunity (Chew & Park, 2004). Several studies have shown that supplementation with carotenoids in the diet may affect the strength of immune responses (Bendich & Shapiro, 1986; Chew *et al.*, 2000; Fenoglio *et al.*, 2002; McGraw & Ardia, 2004). However, there are several reasons why the effect may not be simply dose-dependent, such that an

increase of dietary carotenoids always results in increased immunity (Blount, 2004). First, immune function is not necessarily limited by carotenoids in birds, which have much higher carotenoid levels than, e.g. mammals (Hill, 1999), and especially not in pheasants which have high levels of carotenoids in tissue compared with many other birds (Karadas *et al.*, 2005). Secondly, carotenoids may vary in their immune-modulatory effect. Several studies have demonstrated that canthaxanthin (the carotenoid used in the present experiment) is a potent antioxidant (Palozza & Krinsky, 1992; Surai *et al.*, 2003) that may positively affect immune responses (Bendich & Shapiro, 1986; El-Hafeez *et al.*, 2000; Chew & Park, 2004). But it has also been suggested that other carotenoids such as astaxanthin may exhibit stronger effects on oxidative stress (Miki, 1991; Woodall *et al.*, 1996) and some studies have found weak or no effects of canthaxanthin on immunity (Sklan *et al.*, 1989; Haq *et al.*, 1996; OkotieEboh *et al.*, 1997). However, in an earlier study (Ohlsson *et al.*, 2003), we used food supplemented with carotenoids from *Capsicum* fruits containing an array of different carotenoids (Deli *et al.*, 1996) and still found no effect on pheasant humoral immunity. Finally, it has been suggested that oxidation of carotenoids produces harmful products, which is why a positive direct effect of carotenoid supplementation on immune function is unlikely (Hartley & Kennedy, 2004). Thus, in birds there may be no simple positive effect on immune function of increased intake of carotenoids.

Even if it presently is difficult to ascertain whether carotenoids function as antioxidants *in vivo* (Halliwell & Gutteridge, 1999) and the effect of carotenoids on immunity is inconsistent (Hill, 2002), they may still function as signals of superior health of males (Houde & Torio, 1992; Hartley & Kennedy, 2004). If carotenoid-dependent ornaments are susceptible to oxidative stress and if the activation of the immune system results in the production of free radicals, a high level of carotenoids in ornaments may signal male health (Hartley & Kennedy, 2004). Several studies have shown that activation of the immune system may lower plasma carotenoid levels (Alonso-Alvarez *et al.*, 2004; Peters *et al.*, 2004) including levels of canthaxanthin (Koutsos *et al.*, 2003a). This is compatible with findings that more ornamented males handle infections better (Hill & Farmer, 2005) and that diseased males produce less red ornaments (Brawner *et al.*, 2000; McGraw & Hill, 2000; Hill *et al.*, 2004). Future studies have to show if the occurrence of diseases and parasites in pheasants has any effect on the deposition of carotenoids in the wattle.

Variation in the effect of noncarotenoid nutrition

Although the strength of the humoral immune response was weakly affected by dietary protein, we found cellular but not humoral immunity to relate to male body condition measured as residual mass. If heritable susceptibility against diseases and parasites caused males to vary in body condition, this might be a mechanism that could result in honest advertisement of immune function. However, this requires ornaments to also be sensitive to male body condition. Spur-length was unrelated to our effort to manipulate male body condition, but may be affected by nutritional variation at different time periods or over longer periods of time (cf. Wittzell, 1991). A positive relationship between carotenoid dependent ornament expression and body condition (Hill & Montgomerie, 1994) may arise because nutrition directly affect assimilation or modification of carotenoids (Hill, 2000) or may be caused by correlations with a third factor, such as male foraging ability. Hill experimentally demonstrated that the energetic condition affected ornamentation in house finches (Hill, 2000), and food restricted American goldfinches (*Carduelis tristis*) incorporated less carotenoids into feathers and grew less colourful plumage (McGraw *et al.*, 2005). The difference between our study and those of Hill (2000) and McGraw *et al.* (2005) may be caused by the different nutritional manipulations performed. In our study protein levels varied, but males had *ad libitum* access to food. Thus, birds may be able to adjust for the different nutritional value of food by adjusting the volume of food eaten. We found a positive relationship between body condition and the increase in ornamental coloration in the high treatment group, which may be caused by body condition positively affecting assimilation of canthaxanthin. However, as there was a positive effect of carotenoid treatment on body condition, the causality may also be the other way around. Thus, studies in which the effect of variation of different aspects of nutrition including energy content on carotenoids ornaments are needed.

Conclusion

Our results suggest that wattle coloration may signal male body condition, but there was no evidence that an immune-stimulatory effect of carotenoids resulted in wattle coloration honestly signalling immune function. In contrast, spur length may relate to some aspects of immune function, but we could not show any effect of carotenoid treatment on spur growth. Pheasant ornaments may, however, still honestly signal male immune function. Even if carotenoids do not affect immunocompetence positively, oxidative stress caused by immune responses to parasites and pathogenic micro-organisms may negatively affects plasma and tissue carotenoid levels and thereby the expression of ornaments. Future studies should measure the effect of important diseases and parasites in the wild on pheasant ornamentation.

Acknowledgments

This study was supported by a grant from the Swedish Research Council to H. Smith and from the Swedish

Research Council for Environment, Agricultural Science and Spatial Planning, the Carl Trygger Foundation and the Crafoord Foundation to D. Hasselquist. We thank J. Grindstaff for reading earlier drafts of this manuscript. The study was approved by the Malmö/Lund ethical committee for animal research.

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Received 6 April 2006; revised 12 June 2006; accepted 18 June 2006