



Male coloration reveals different components of immunocompetence in ostriches, *Struthio camelus*

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It has been suggested that secondary sexual ornamentation signals male ability to resist infections, as only high-quality individuals are able to invest both in high immune defence and elaborate ornament expression. Such ornaments could thus serve as indicators of male quality and could be used by females in choosing mates. Ostriches are sexually dimorphic with regard to coloration of their feathers, bill, neck and legs, and have a promiscuous mating system, with a high degree of reproductive skew, particularly in males. We investigated the relationship between the coloration of the feathers, bill, neck and legs of 15 male ostriches, maintained in a breeding flock, and the cell-mediated (measured using a phytohaemagglutinin (PHA) injection) and humoral components of their immune systems, as well as their heterophil:lymphocyte ratio. We found that male responses to PHA injection and humoral responses to tetanus were predicted by leg coloration, humoral responses to diphtheria were predicted by white feather coloration, and the heterophil:lymphocyte ratio was related to bill coloration. These traits, which relate to male immune capacity, are exposed during male–male interactions and courtship display, so we suggest that these visual cues could provide valuable information on male quality to females (as well as rival males), forming the basis of mate choice in this species.

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Females of many species prefer to mate with males displaying the most elaborate ornamentation. Under the assumption that the expression of these traits is condition dependent, only males in prime condition will be able to develop the most exaggerated ornamentation (Andersson 1994), thereby revealing either direct or indirect genetic benefits that a female could obtain by mating with a given male. For instance, ornaments may reflect general condition (Goransson et al. 1990), the ability to forage for food (Slagsvold & Lifjeld 1988; Senar et al. 2002) or the ability to cope with parasites (Hamilton & Zuk 1982; Møller & Saino 1994). In birds, females frequently use sexually dimorphic characteristics to discriminate between males during mate choice (Andersson 1994; Saino et al. 2002). Furthermore, several studies have shown that sexually dimorphic traits such as feather characteristics (length of tail feathers: Møller & Petrie 2002; plumage coloration: Gonzalez et al. 1999; Doucet et al. 2004), beak coloration (Faivre et al. 2003) and

spurs (Ohlsson et al. 2002) also reflect the male's ability to raise an immune response against novel antigens.

Immune function often shows strong condition dependence with only individuals in good condition (as illustrated by the expression of ornamentation) being able to produce strong immune responses. Norris & Evans (2000) defined immunocompetence as the ability of a host to prevent or control infection by pathogens and parasites, and variation in immunocompetence is assumed to represent general individual disease resistance. Consequently, females basing their mate choice decisions on ornamentation could acquire males with better resistance to parasites, resulting in either direct benefits in species with paternal care, and/or indirect genetic benefits when offspring inherit genes for superior immunocompetence (Folstad & Karter 1992; Andersson 1994; Westneat & Birkhead 1998). In accordance with this, Bonneaud et al. (2005) showed that a specific MHC allele was associated with higher responses to two different T-cell-dependent antigens: phytohaemagglutinin (PHA, which is cell-mediated) and sheep red blood cells (which are mediated humorally).

Ostriches are promiscuous, and in the wild both males and females have multiple partners (Bertram 1992; Kimwele & Graves 2003). The ostrich communal nesting system is unique in that the major female allows minor females to lay in her nest, even though they provide no parental care. Only the major female and major

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male provide parental care in the form of incubation and guarding the offspring until independence. Furthermore, a remarkable feature of cohorts is that chicks differ greatly in size, and these size differences are likely to have a genetic basis arising from parental genotypic differences. Ostriches are sexually dimorphic; females have a dull-brown plumage while males have a black plumage with some white feathers, as well as coloured bill and legs (Deeming 1999). Both males and females use a repertoire of visual displays, in several of which the wings play a major part. For instance, wings are used during aggressive encounters with predators or opponents, in which ostriches raise both wings high above the body, or flick them alternately up and down beside the body. Most importantly, wings are involved in the courtship display (or 'kantling' behaviour), whereby the male typically sits on his legs, while his wings are held forward, directly exposed to the females, and his neck swings from side to side (Bertram 1992). The kantling display is also used during antagonistic interactions between males, and is usually performed by a male who is driving a competitor away. Furthermore, during the breeding season the bare shins and the beak of ostrich males change from light pink to crimson red (Lambrechts 2004). Females are able to discriminate between males and to invest differently at the egg stage with respect to the coloration of male traits involved in the kantling display (Bonato et al. 2009). Although the extent to which male traits reflect individual condition remains unknown, chick size variation could potentially be explained by females choosing and/or investing in males of a higher quality (i.e. with elaborate ornaments and/or higher immunocompetence), thereby enhancing offspring fitness.

Our aim in this study was to examine whether male traits (specifically coloration) reflect a male's ability to raise an immune response. Because of the complexity of the immune system, stimulation of more than one component of the immune system is required (Sheldon & Verhulst 1996; Norris & Evans 2000; Viney et al. 2005) to elucidate potential trade-offs between traits and immunocompetence. We assessed cell-mediated and humoral immunocompetence with challenge tests that have been extensively used in immunology studies. The advantage of these techniques is that individuals are exposed to a standardized challenge to their immune system and the response of the immune system is quantified in a standardized way. In addition, we monitored the heterophil:lymphocyte (H:L) ratio at the time of sampling, which provides a crude estimate of an individual's current immune status (Davis et al. 2008). Furthermore, avian vision differs from that of humans in several ways: birds are sensitive to the ultraviolet (UV) part of the spectrum (320–400 nm) to which humans are blind (Bowmaker 1980; Bennett & Cuthill 1994), and have four cone types, rather than three as found in humans, implying that birds have the potential for tetrachromatic vision (Chen & Goldsmith 1986; Jane & Bowmaker 1988; Bowmaker et al. 1997; Wright & Bowmaker 2001). Therefore, we used UV-visible range spectrophotometry to measure accurately the colour of the bill, neck, feathers and legs of 15 males maintained within a breeding flock. In particular, the white feathers, and pink bill and legs, are particularly conspicuous during the breeding season, and we suggest that male ostriches use multiple signals to advertise their quality to both male competitors and females, thereby forming the basis of sexual selection in this species.

METHODS

Study Population

We carried out the study on 15 South African black ostrich males, *S. camelus* var. *domesticus*, maintained at a research farm in Oudtshoorn, South Africa, from August 2005 to March 2006. The

breeding flock consisted of two groups, each in an 8 ha camp, containing a ratio of male:female individuals of 7:12 and 8:11, respectively. Males and females were all of breeding age (range 2–5 years). Food and water were provided twice a week. Diets were formulated according to the nutrient requirements of the birds at the specific stage of growth (Brand & Gous 2006). Males selected for this experiment were roughly of the same height, and were weighed before the experiment using an electronic balance (Rudd, Pomona, South Africa). We recorded colour measurements on the birds in November 2005, in the middle of the breeding season, when the bare shins and the bill of ostrich males develop a typical colour, ranging from light pink to crimson red (Lambrechts 2004). The cell-mediated (together with blood samples to estimate the H:L ratio) and the humoral immune assays were performed a month apart in December 2005 and January 2006, respectively, for practical reasons and to minimize interaction between the different treatments. Individuals were carefully restrained by hand during the colour measurements and the injections. Three trained technicians held the bird while another injected the PHA or the diphtheria and tetanus vaccine (see below). The same method was used for the blood sampling. The procedures lasted between 5 and 10 min and we verified that the bird resumed normal behaviour a few minutes afterwards. We also verified that the injections did not cause visible wounds or infections during the following days. We observed no negative effects of the injections in our study. Ethical clearance for this work was granted by the Stellenbosch University ethics committee.

Colour Measurements

Reflectance spectra between 300 and 700 nm were recorded using an Ocean Optics USB 2000 spectrophotometer and a PX-2 xenon lamp (Ocean Optics, Dunedin, Florida, U.S.A.) on five traits (bill, neck skin, black feathers, white feathers and legs) on each male. As each trait appeared uniform in colour, it was measured 10 times in randomly allocated places. Reflection was recorded using a probe held normal to the surface, collecting light from a spot of 6 mm in diameter. A white reference (Spectralon 99% white standard) and a dark reference for calibration were taken before measuring each individual trait.

Immune Assays

We estimated two components of immunocompetence: the T-cell-mediated immune response and the B-cell humoral response. In addition, we recorded the H:L ratio at the same time as we conducted the T-cell-mediated immune response assay, to provide a crude estimate of the bird's current immune status.

Cell-mediated immunity was challenged by using a phytohaemagglutinin (PHA) injection. Although this is a standard method of assessing cell-mediated immunity in poultry (Cheng & Lamont 1988), a recent study on house sparrows, *Passer domesticus*, has shown that PHA swelling is more complex than previously thought: it is correlated with cell-mediated components of the immune system, but not exclusively so, as some of the swelling is attributable to other aspects of immune function, both innate and adaptive (Martin et al. 2006). Our results should be interpreted in this context. Males were inoculated subdermally with 0.4 mg of a PHA solution (Sigma, Pomona, South Africa; L-8754) dissolved in 0.04 ml of phosphate buffered saline (PBS) in the right wing web, and with 0.04 ml of PBS in the left wing, as a control test. We measured the thickness of the wing webs on three occasions; before the injection, 6 h and 24 h later. On each occasion we used a digital calliper and measured the thickness of the wing web three times and used the average of these measures. Repeatabilities of

measurements (Lessells & Boag 1987) were 0.79 and 0.83 for the right and left wing, respectively. The PBS control injection was administered despite the recommendation of Smits et al. (1999) as we observed slight swellings at the point of injection (6 h: $\bar{X} \pm SD = 1.04 \pm 0.36$ mm; 24 h: 1.33 ± 0.39 mm). The wing web swelling was calculated as the difference in thickness of the PHA-injected versus the PBS-injected wing, which indicates the strength of the response to the PHA injection.

To measure the B-cell-mediated humoral response, we elicited an antibody response in the birds by injecting a solution of 0.5 ml of a diphtheria–tetanus vaccine (DTVAX) in their neck. As the concentration of a dose of this vaccine (0.5 ml) is calculated for an adult human, we used the same concentration for our adult ostriches, which had a mean mass $\pm SD$ of 115.60 ± 12.33 kg. We collected 100 μ l of blood from the right external jugular vein before the injection, and 10, 14, 21 and 30 days postinjection. After centrifugation (4000 rpm for 10 min), plasma was stored at -70°C . To assess the level of antibodies in the plasma, we then conducted an enzyme-linked immunosorbent assay, ELISA (Hasselquist et al. 1999; Råberg et al. 2003; Hanssen et al. 2004). Briefly, 96-well microtitre plates (Costar, Cambridge, MA, U.S.A.) were coated with either diphtheria or tetanus antigens at 4°C for 24 h. After washing plates and blocking wells with 3% milk powder diluted in 0.01 M PBS/Tween 20, we added diluted plasma samples (see below) to the wells and allowed them to incubate overnight at 4°C . Plates were then washed, and a rabbit–antiostrich Ig antiserum, obtained from Professor Dirk Bellstedt (Department of Biochemistry, University of Stellenbosch, Stellenbosch, South Africa; diluted 1:800), was added to each well. Following 1 h of incubation at 37°C and a wash, peroxidase-labelled goat–antirabbit serum (1:2000 dilution; Sigma A6154) was added to the wells. After a secondary incubation (45 min at 37°C) and a final wash, 2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS; Sigma, A1888) and peroxidase were added to the wells, and the plates were then immediately transferred to a molecular devices V_{max} kinetic reaction ELISA reader. The plates were read every 30 s for 12 min using a 405 nm wavelength filter. Antibody concentrations were calculated according to the slope of substrate conversion over time in units $10^{-3} \times \text{optical densities (OD) per min (mOD/min)}$, with a higher slope indicating a higher titre of antidiphtheria or antitetanus antibodies in the sample. Each plate also included baseline samples collected from birds before injections (negative controls). Plasma samples diluted to 1:400 (for diphtheria antibodies) and 1:800 (for tetanus antibodies) were used in all analyses. All samples were run in duplicate, and the average of the two readings was our measure of antibody levels in the plasma. Blanks with only buffer were also included on each plate. Final measures of antibody levels were expressed as the difference between baseline and post-immunization antibody titres of individuals and were log transformed to obtain a more normally distributed data set and facilitate the use of parametric statistics.

To establish the H:L ratio, we collected blood smears by venipuncture from the wing vein of the birds just before the injection of PHA in the wing web. Blood smears were air dried, fixed in 100% methanol the same day and stained with 5% Giemsa solution. We counted 100 white blood cells on each slide by moving the microscope stage from one field to another and calculated the H:L ratio. High H:L ratios are traditionally considered to indicate stress in poultry (Gross & Siegel 1983).

Statistical Analysis

A principal components analysis (PCA) was performed on the reflectance spectra for all five traits for each male, and this reduced a number of highly correlated variables (reflectance at 2.4 nm

intervals) to a small number of independent variables. The PCA on colour measurements revealed that three principal components explained between 95.8% and 98.9% of the total variance in the five traits measured (Table 1). The first principal component (PC1) summarized between 70.5% and 94.8% of the spectral variation in these traits, whereas the second and third principal components (PC2 and PC3) accounted for between 3.3% and 17.5%, and between 0.8% and 7.8%, respectively. In reflectance spectra of natural objects, PC1 is usually relatively flat, in which case it describes achromatic variation or ‘achromatic brightness’ (Endler & Théry 1996), while PC2 and PC3 describe variation in spectral shape and are indirectly related to hue and saturation (Endler 1990; Bennett et al. 1997; Cuthill et al. 1999).

Responses to PHA injection, antibody titres of the humoral responses and H:L ratios were log transformed to achieve normally distributed residuals. We used a paired *t* test and a repeated measures ANOVA to detect any differences in the strength of the responses across time for the responses to PHA injection and humoral responses, respectively, and we selected the strongest immune responses for the subsequent analysis.

A generalized linear model (GLM) was constructed for each of the immune assays, with camp as a fixed factor, and using male body mass and age as well as principal component scores for the spectrometric measures of each male trait measured as explanatory variables. All variables were initially included, and then dropped until the model contained only significant terms. As four GLMs were constructed, Bonferroni adjustments were used to control for type I errors (Wright 1992), lowering the significance threshold to 0.0125. Statistical analyses were performed using SPSS 16 (SPSS Inc., Chicago, K, U.S.A.).

RESULTS

Immune Assays

The response to the PHA injection was highly variable between individuals and higher 24 h after the injection, although not statistically different from the measurement recorded 6 h post-injection (6 h: $\bar{X} \pm SD = 2.54 \pm 2.14$ mm; 24 h: 3.92 ± 3.64 mm; paired *t* test: $t_{14} = -1.791$, $P = 0.095$). The primary antibody responses for both diphtheria and tetanus were highest 21 days after the injection (repeated measures ANOVA: diphtheria: $F_{1,3} = 44.24$, $P = 0.0002$; tetanus: $F_{1,3} = 28.73$, $P = 0.0001$; Fig. 1) and were only weakly correlated with each other ($r_{14} = 0.398$, $P = 0.071$). Finally, the H:L ratio was also highly variable between individuals ($\bar{X} \pm SD = 0.42 \pm 0.22$). We did not detect any effect of camp on the response to the PHA injection ($F_{1,1} = 0.336$, $P = 0.752$), the humoral response (diphtheria: $F_{1,1} = 0.249$, $P = 0.626$; tetanus: $F_{1,1} = 0.280$, $P = 0.572$) or on the H:L ratio ($F_{1,1} = 0.614$, $P = 0.447$). For subsequent analyses, we used the strongest response to the PHA injection and the strongest humoral response (24 h post-injection, and 21 days postinjection, respectively). When analysing intercorrelations between these immune assays, we found none of them correlated with each other ($P > 0.05$).

Table 1

Principal components analysis for the colour measurements of the bill, neck, black feathers, white feathers and legs of 15 male ostriches

Trait	Principal components (% of variation explained)			
	PC1	PC2	PC3	Total
Bill	87.4	6.1	3.4	96.9
Neck	89.8	6.3	2.3	98.4
Black feathers	70.5	17.5	7.8	95.8
White feathers	94.8	3.3	0.8	98.9
Legs	90.0	5.5	3.0	98.5

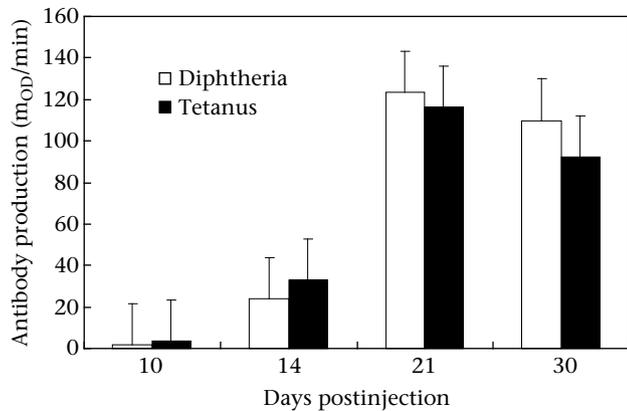


Figure 1. Antibody production ($10^{-3} \times$ optical densities per min (m_{OD}/min)) to a diphtheria-tetanus vaccine in 15 male ostriches over 30 days after injection.

Immune Function and Colour Measurements

The immune responses were not predicted by male age or body mass ($P > 0.05$), but by the principal component values of bill, white feathers and legs (Table 2, Fig. 2). The H:L ratio showed a quadratic relationship with PC2 of the bill ($F_{1,14} = 6.974$, $P = 0.010$), and the response to the PHA injection showed a positive relationship with PC2 of the legs ($F_{1,14} = 3.392$, $P = 0.004$). For the humoral assay, the diphtheria response showed a positive relationship with PC2 of the white feathers ($F_{1,14} = 2.466$, $P = 0.010$), whereas the tetanus response showed a quadratic relationship with PC3 of the legs ($F_{1,14} = 4.164$, $P = 0.002$).

The reflectance spectra for these traits were similar to those reported for other bird species (Fig. 3). The white feathers spectrum rose gradually from 300 to 500 nm, above which it was uniformly high and flat (see Mennill et al. 2003; Shawkey & Hill 2006), while the reflectance spectra of the bill and the legs also rose gradually, but flattened out to a maximum reflectance above 600 and 650 nm, respectively (see Pryke et al. 2002; Bolund et al. 2007). PC1 for all

these traits was relatively flat across the spectra (Fig. 4), and therefore represents 'achromatic brightness' (Endler & Théry 1996). PC2 and PC3 were not spectrally flat, and thus represent chromatic variation (hue and saturation) of these traits: only those trait components predicting immune responses (Table 2) are described in detail here. PC2 of the bill was high and negative at shorter (300–400 nm) and longer wavelengths (575–700 nm), and slightly high and positive at intermediate wavelengths (400–575 nm). PC2 of the legs was high and negative at shorter wavelengths (300–425 nm), with a peak at 350 nm, and at longer wavelengths (575–700 nm) and slightly high and positive at intermediate wavelengths (425–575 nm). PC3 for the legs was also high and positive at shorter wavelengths (300–350 nm) and high and negative at longer wavelengths (600 and 700 nm). All three components thus represent contrasts between UV and 'redness'. By comparison, PC2 for white feathers was low and negative at shorter (300–340 nm) and longer (575–700 nm) wavelengths and showed a peak reflectance at intermediate wavelengths (440–575 nm), representing the 'green' part of the spectrum.

DISCUSSION

Our findings revealed that different male traits reflect different components of the immune system. We found that males had: (1) a higher response to the injection with higher values of PC2 of the legs; (2) a higher humoral response to diphtheria with higher values of PC2 of the white feathers and higher humoral response to tetanus for intermediate values of PC3 of the legs; and (3) a lower H:L ratio with intermediate values of PC2 of the bill. These findings are consistent with the multiple signalling hypothesis (Møller & Pomiankowski 1993), which posits that multiple signals in sexual selection reflect different aspects of individual quality. For instance, in the pheasant, *Phasianus colchicus*, females show a preference both for spur length (which reflects condition and viability: Gornansson et al. 1990) and for male display activity, which is correlated with parasite load (Johnstone 1995).

Table 2
Factors influencing the cell-mediated response (PHA response), the humoral response (diphtheria and tetanus antibody responses) and the heterophil:lymphocyte ratio (H:L) in 15 male ostriches

Variable	PHA response			Diphtheria response		Tetanus response		H:L		
	df	F	P	F	P	F	P	F	P	
PC1 scores for										
Black feathers	1,14	0.570	0.505	1.762	0.316	1.613	0.332	0.240	0.645	
White feathers	1,14	0.063	0.818	1.847	0.245	0.004	0.957	0.681	0.447	
Legs	1,14	1.768	0.195	1.903	0.272	1.342	0.366	0.005	0.945	
Neck	1,14	2.068	0.069	0.052	0.842	1.030	0.385	0.010	0.926	
Bill	1,14	0.739	0.439	2.430	0.092	0.946	0.433	0.113	0.750	
PC2 scores for										
Black feathers	1,14	1.074	0.110	1.932	0.228	0.405	0.590	0.386	0.561	
White feathers	1,14	1.090	0.092	2.466	0.010	0.713	0.487	0.913	0.383	
Legs	1,14	3.392	0.004	0.448	0.572	1.580	0.336	0.239	0.645	
Neck	1,14	0.391	0.576	0.506	0.518	1.198	0.354	0.569	0.484	
Bill	1,14	1.087	0.374	2.340	0.104	0.593	0.522	6.974	0.010	
PC3 scores for										
Black feathers	1,14	0.323	0.609	0.421	0.583	1.631	0.330	1.759	0.255	
White feathers	1,14	0.824	0.406	0.005	0.951	0.720	0.552	0.996	0.375	
Legs	1,14	0.608	0.471	1.957	0.210	4.164	0.002	1.462	0.293	
Neck	1,14	0.003	0.961	1.953	0.212	2.991	0.182	0.177	0.691	
Bill	1,14	0.011	0.922	2.325	0.105	0.793	0.467	2.103	0.221	
Male mass	1,14	0.867	0.388	1.997	0.257	0.295	0.642	2.458	0.192	
Male age	1,14	0.404	0.548	0.144	0.741	1.166	0.393	0.711	0.548	
Camp	1,1	0.336	0.752	0.249	0.626	0.28	0.572	0.614	0.447	

Significant values ($P < 0.0125$) are in bold.

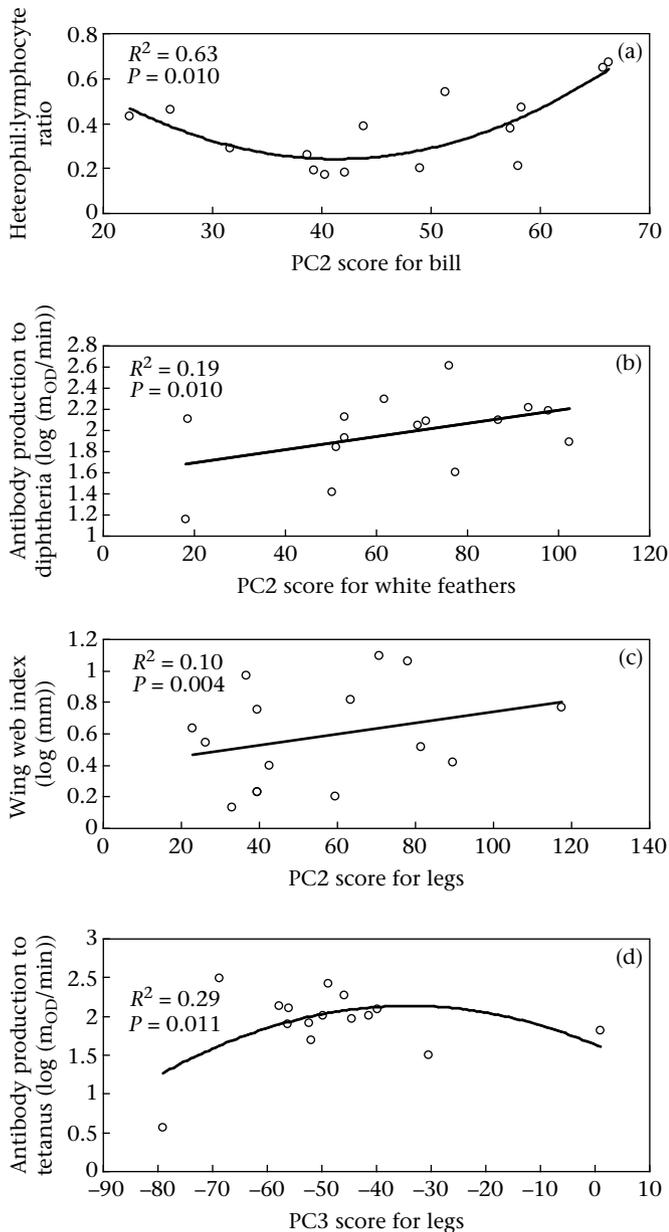


Figure 2. Components of the immune system in relation to the principal component coefficients derived from reflectance spectra for (a) the bill, (b) the white feathers, (c) and (d) the legs of 15 male ostriches. (a) Heterophil:lymphocyte ratio on the day of the injection, (b) antibody production to diphtheria measured as $10^{-3} \times$ optical densities (m_{OD}/min), (c) wing web index 24 hours postinjection and (d) antibody production to tetanus (m_{OD}/min).

For the traits we measured, PC2 (the component explaining most of the colour variation) of the white feathers showed peak reflectance between 400 nm and 575 nm. Wright & Bowmaker (2001) demonstrated that ostriches are most sensitive to these intermediate wavelengths, with their four spectral cone classes having a maximum absorbance between 405 nm and 570 nm. As these traits reflect in the area of maximum absorbance of the bird's visual spectrum, they could act as amplifiers whereby high-quality males are able to advertise their condition optimally. As male ostriches use a variety of displays to communicate and interact with females and/or other males (Bertram 1992), these traits could serve both to attract potential mates (via the courtship display where white feathers are exposed) and to deter other males or predators from engaging in antagonistic interactions.

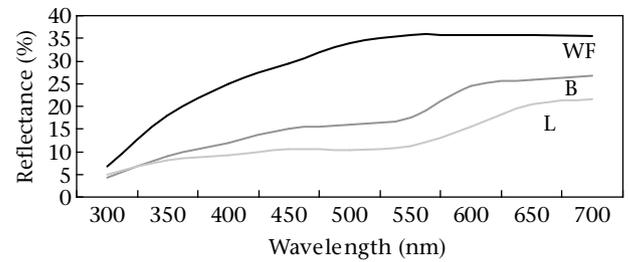


Figure 3. Spectral reflectance of the bill (B), white feathers (WF) and legs (L) of 15 male ostriches.

Our results showed that males that are able to maintain trait coloration are able to raise immune responses. We found that the response to the PHA injection was higher in males with higher values for PC2 of their legs. This positive relationship between the response to the PHA injection immunity and leg coloration may be strategically important for male ostriches, especially during the breeding season because of competition and conflicts over territories and/or females. As legs are mostly used to strike opponents and small predators (Bertram 1992), they are more prone to injuries. Thus, the cell-mediated immunity may be mobilized to heal these injuries and consequent infections, and may be particularly important for male ostriches during the breeding season. In accordance with this, the bare shins of ostrich males change in colour from light pink to crimson red as males become territorial, and continuing into the breeding season (Bertram 1992; Lambrechts 2004).

The analysis of the humoral response revealed an interesting feature, in that responsiveness to the two antigens reflected different traits. The humoral response to diphtheria was higher in males with higher PC2 values for white feathers, and the humoral response to tetanus was higher with intermediate PC3 values for the legs.

The positive relationship between the coloration of the white feathers and the primary response to diphtheria is of particular interest as it suggests that white feathers are condition dependent. Female ostriches lay heavier eggs in response to male traits involved in the courtship display (Bonato et al. 2009), with PC2 of the white feathers predicting egg mass more strongly than any other trait. No studies to date have investigated whether the coloration of ostrich white feathers could increase predation risk, but males and females specifically use white feathers in attempting to distract predators from chicks (Bertram 1992), underlying the conspicuousness of this trait. A male's ability to display and maintain a contrast between its black and white feathers could provide the female with valuable information on the male's condition, in particular its ability to raise an immune response, acting as a signal which influences the degree of her investment in eggs. Similarly, in female eiders, *Somateria mollissima*, the width of the white wing bars reflects previous immune challenges, and could therefore potentially be used as an honest signal of individual quality (Hanssen et al. 2008).

The finding of higher primary immune responses to tetanus at intermediate levels of PC3 in leg coloration suggests that this component of defence has not only fitness benefits but also costs (Viney et al. 2005), and the expression of this trait may reflect variation in how individuals trade off these costs and benefits. Studies on blackbirds, *Turdus merula* (Faivre et al. 2003) and red junglefowl, *Gallus gallus spadiceus* (Zuk & Johnsen 1998) have demonstrated that males pay a cost for the expression of a secondary sexual character by decreasing investment in one of the components of the immune system. For instance, in blackbirds,

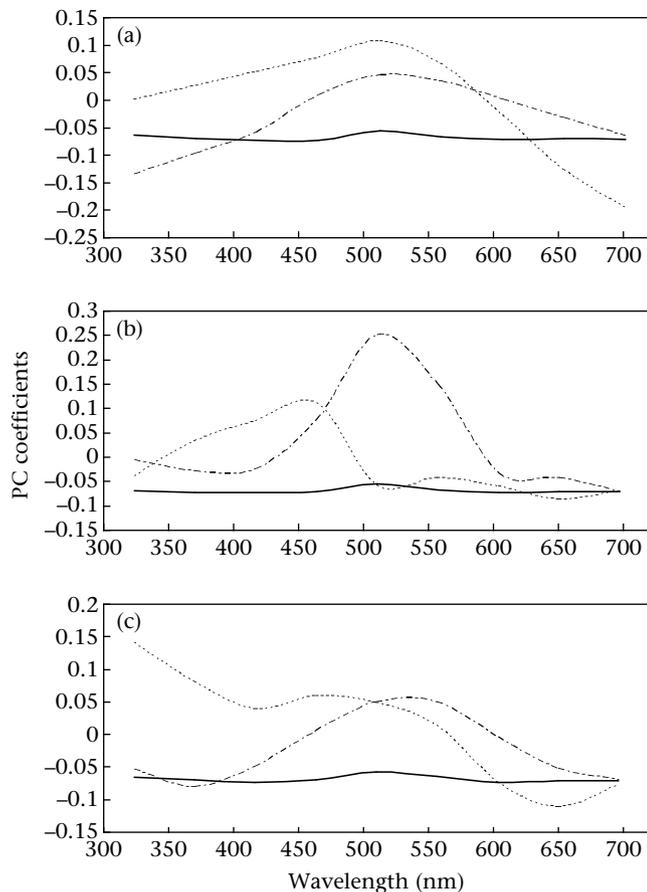


Figure 4. Principal component scores derived from the reflectance spectra for three body regions of 15 male ostriches: (a) bill, (b) white feathers, (c) legs. Solid lines indicate PC1; dashed lines: PC2; dotted lines: PC3. Fig. 4b is reprinted from Bonato et al. (2009), with permission of Elsevier.

males with more orange bills had high cellular immunocompetence while their humoral immunocompetence was compromised (Faivre et al. 2003). In our study, as the cell-mediated response also affected leg coloration, the maintenance of this specific component of the immune system might have significant effects on the maintenance of humoral responses.

A precondition of condition-dependent models of immune trade-offs is that multiple components of the immune system should be intercorrelated (Westneat & Birkhead 1998). Recent studies have found contrasting results: in some avian species different components of immunity showed correlated responses (Westneat et al. 2003; Ekblom et al. 2005) whereas in others they did not (Zuk & Johnsen 1998; Faivre et al. 2003). We only found a weak positive relationship between humoral responses to diphtheria and tetanus, while there were no relationships between these and either responses to the PHA injection or H:L ratios, which in turn were unrelated to each other. However, we measured only primary immune responses, as the disturbance caused to the breeding flocks did not allow additional measurements. Variation may exist in the quality of immunological memory, which determines the strength of the secondary immune response: as the secondary response is stronger, variation between individuals could be greater and have more impact on their health.

Furthermore, the quadratic relationship between H:L ratio and bill coloration suggests that only individuals with intermediate PC2 values have a low H:L ratio. The H:L ratio is widely used as a stress estimator in poultry (Gross & Siegel 1983; Maxwell 1993) and is

known to increase in response to various stress factors, such as infections or disturbance. Although this signal could inform females and/or competitors of the general condition of an individual, the use of the H:L ratio should be interpreted with care, as it could indicate either an immunocompetent individual or an individual currently fighting an infection (Sheldon & Verhulst 1996; Norris & Evans 2000).

Immunocompetence in birds appears to be affected by the general condition of the individual, which in turn could be reflected in male size, the expression of ornamentation or territory quality (Norris & Evans 2000; Møller & Petrie 2002; Blount et al. 2003), all of which could potentially inform female mating decisions. Studies on the effect of testosterone on the expression of sexual ornamentation, immunity and mating strategies have led to contrasting results across species (Roberts et al. 2004). In male ostriches, the level of testosterone increases with the level of aggression between territorial males during the breeding season (Degen et al. 1994). As male aggression is characterized by forward kicking and kantling behaviour displayed to other males (Bertram 1992), and as the shins of ostrich males change in colour during that time (Lambrechts 2004), this emphasizes the crucial role of both legs and white feathers. Further studies should be conducted to determine whether testosterone could influence the degree of the immune response observed, as well as the expression of these specific male traits.

Coloration in birds is essentially derived from two types of pigmentation, melanins and carotenoids. Melanins are responsible for most black, brown and brick-red coloration, are cheap to produce and can be synthesized by the organism (Maynard Smith & Harper 1988). The major component of melanins is the amino acid tyrosine, which is also an important precursor in immunological processes (Owens & Wilson 1999). Carotenoids, by contrast, are responsible for most bright yellow, orange and red coloration and are exclusively acquired through the diet (Brush 1990). They also play an important role in many immunological and metabolic pathways, as their expression can be affected by parasitism (Lozano 1994). In particular, manipulation of melanin (McGraw et al. 2003) and carotenoid (Blount et al. 2003) concentration in other bird species has demonstrated the relationship between these pigments, mate quality and consequently sexual attractiveness. For instance, modulation of carotenoid supplies in zebra finches, *Taeniopygia guttata*, showed that females chose to mate with carotenoid-rich males displaying redder bills and a higher cell-mediated response (Blount et al. 2003). The limitation of our study is that we were unable to manipulate the concentration of the pigments responsible for trait coloration, something which could usefully be investigated in further studies.

In conclusion, the coloration of the bill, white feathers and legs appears to reflect different components of the immune response of ostrich males. High-quality males appear to be able to maintain these signals but also to maintain both cell- and humorally mediated components of their immune system. These findings are consistent with the hypothesis that multiple ornaments signal different aspects of male quality. Our results explain patterns observed in maternal investment in ostriches, and could be used by the ostrich farming industry in selecting breeding males.

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