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Carotenoid-based bill colour as an indicator of immunocompetence and sperm performance in male mallards

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Abstract

Female mate choice is often based on exaggerated sexual traits, signals of male qualities that females cannot assess directly. Two such key qualities are male immune and/or sexual competence, whereby honesty in signalling could be maintained by physiological trade-offs. Carotenoid-based ornaments likely constitute such honest signals, as there is direct competition for (limited) carotenoids between ornament deposition and anti-oxidant support of immune or sperm functioning. Using spectrometry, we assessed the potential signalling function of the yellow, carotenoid-based colour of the bill of male mallards, a target of female mate choice. Here we demonstrate that bill reflectance varied with plasma carotenoid level, indicating antioxidant reserves. Moreover, lower relative UV reflectance during autumn pairing predicted immune responsiveness and correlated positively with sperm velocity during breeding, a trait that affects fertility. Our data provide support for current theories that females could use carotenoid-based sexual signals to detect immune vigour and fertilizing ability of prospective mates.

Introduction

Males of many species show a variety of exaggerated ornamental traits that are sexually selected through female mate preferences. It is commonly assumed that these ornaments signal male qualities to females, which they cannot assess directly (Andersson, 1994). When costs of trait development and/or maintenance enforce honesty on ornament exaggeration, females may obtain direct and/or indirect fitness benefits by basing their mate choice on such traits (Johnstone, 1995). Two key aspects of male quality that ornaments have been hypothesized to convey are immune competence, the ability to deal with parasites and diseases (Hamilton & Zuk, 1982; Zuk & Stoehr, 2002), and sexual competence, that is male fertilizing ability (Trivers, 1972; Sheldon, 1994). For signals of immune functioning (Folstad & Karter, 1992; Lozano, 1994; Westneat & Birkhead, 1998; von Schantz *et al.*, 1999; Blount *et al.*, 2001), as well as male fertility (Folstad & Skarstein, 1997; Hillgarth *et al.*, 1997), similar hormonal and nutritional trade-offs have been proposed to maintain honesty of ornament expression. A principal physiological link between level of ornamentation, immunocompetence and sperm quality is formed by carotenoids, plant pigments that are limited in animals but fulfil several competing key physiological functions (Olson & Owens, 1998).

Carotenoids determine the red, yellow, and orange colour of many secondary sexual characters, such as fleshy ornaments and colourful plumages, which are among the most familiar targets of female choice (Olson & Owens, 1998; Hill, 2002). Animals cannot synthesize carotenoids but must ingest them with their food (Olson & Owens, 1998), and ultimately carotenoid intake limits ornament expression (Hill *et al.*, 2002). Accordingly, carotenoid supplementation studies have almost universally shown that additional dietary carotenoids can enhance ornament coloration (Kodric-Brown, 1989; Hill, 1992; Saino *et al.*, 2000; Blount *et al.*, 2002, 2003, but see Fenoglio *et al.*, 2002). However, carotenoids are not only deposited in ornaments, they are also important antioxidants owing to their ability to scavenge free radicals and quench reactive oxygen metabolites (Bendich, 1993; Mortensen *et al.*, 1997). These highly reactive molecules are by-products of the metabolism that can cause extensive oxidative damage ('oxidative stress', von Schantz *et al.*, 1999). Free radicals are generated in high

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concentrations by metabolically highly active tissues, such as activated immune cells and organs and the testes, and these therefore require high levels of antioxidant protection (von Schantz *et al.*, 1999; Blount *et al.*, 2001).

Antioxidant properties of carotenoids make them powerful immunostimulants, and ample evidence exists that carotenoid supplementation can enhance immunity (for review see Bendich, 1989; Blount *et al.*, 2003). Thus, competition for the available pool of carotenoids between ornaments and immune function may confer honesty on sexual signals (Lozano, 1994; von Schantz *et al.*, 1999). Indeed, carotenoid-based plumage (Saks *et al.*, 2003b) and to a lesser extent bill colour (Faivre *et al.*, 2003b) have been shown to reflect immune capacity, while experimental immune activation resulted in reduced expression of carotenoid-dependent coloration (Saino *et al.*, 2000, 2003; Faivre *et al.*, 2003a). Besides such roles in coloration and immunity, the antioxidant activity of carotenoids may be crucial to protect sperm from oxidative damage (von Schantz *et al.*, 1999; Blount *et al.*, 2001). Sperm production and activity generate high levels of free radicals and sperm is particularly vulnerable to oxidative stress (Gagnon *et al.*, 1991). Accordingly, antioxidants present in semen reduce the susceptibility of sperm to oxidation and increase fertility (Blount *et al.*, 2001). Evidence that sexual ornamentation may indicate male fertility is so far rather limited (Blount *et al.*, 2001; Evans *et al.*, 2003), while no studies appear to have examined the interactions between carotenoid availability, ornamentation, and sperm quality.

We investigated the relationships between carotenoid-based sexual signalling, immunocompetence, and sperm quality in mallard ducks, *Anas platyrhynchos* L. Mallards are seasonally sexually dimorphic and males in colourful nuptial plumage have a bright yellow bill that contains carotenoid pigments (Lönnberg, 1938; Cramp & Simmons, 1977; S. Andersson & A. Johansson pers. comm.). Male plumage and bill become brown and female-like during the post-breeding moult in mid summer, and return to their ornamental state before autumn. Pair formation in resident mallards takes place in autumn, with pairs remaining together until breeding the next spring (Williams, 1983). During mate choice, females show a strong preference for males with a more yellow bill (Omland, 1996a,b). We objectively assessed brightness and spectral shape (colour) of the bill by reflectance spectrometry. During the autumn pairing season we investigated if bill reflectance correlated with male condition, plasma carotenoid level and immunocompetence, estimated as antibody production to a single immunization with a nonpathogenic immune stimulus (sheep red blood cells). In addition, we investigated whether bill colour could provide information on male sperm quality during mating. Our data show that spectral shape varied with plasma carotenoid level, immune responsiveness as well as sperm swimming performance.

Materials and methods

Animals and design

Free-living mallard duck pairs were collected from the wild in Bavaria, Germany in 2000. Eggs laid by those pairs in 2000 and 2001 were artificially incubated and ducklings raised in large groups. Experimental ducks belonged to 13 families, each with 1–5 (median = 3) full brothers. During the breeding season (February to June) drakes were housed with one female, or in isolation when used for sperm collection (see below). Outside the breeding season they were housed in three mixed-sex flocks (sex ratio 1 : 1) in outdoor aviaries (60, 60 and 120 m^2 with 11, 12 and 17 males, respectively). In August 2002, all females were redistributed while males remained in their original aviaries. Water was provided in a concrete pond of 4 m^2 plus two bathing pools or a fenced section (70 m^2) of a natural lake. Commercial duck food (Anseres III, Kaspar Faunafood, Netherlands containing 14% protein, 4% fat, 5% fibre, 7% ash, 20 IE g^{-1} vitamin A, 4 IE g^{-1} vitamin D and 20 mg kg^{-1} vitamin E) and wheat were provided *ad libitum* at five or more feeding stations per aviary, with regular addition of lettuce. Birds were habituated to daily human presence in their aviaries and accustomed to regular capture.

During the autumn courtship and pairing season of 2002, male condition, immunocompetence, plasma carotenoid levels and yellow bill colour were assessed. Birds were captured on 21 and 22 October after the moult into ornamental plumage had ceased in all males. All birds in one aviary were captured in one session. Blood samples were taken in heparinized capillary tubes and immediately placed on ice until centrifugation and collection of plasma. Plasma was frozen at −70 °C until analysis of antibodies, carotenoids and protein (see below). To assess body condition we measured body mass (to 0.1 g). As an indicator of structural body size we used the first principal component of bill width and length, head-bill length and tarsus length, which accounted for 85% of variation in skeletal size. As body fat is not necessarily a good indicator of condition in captive birds, we additionally determined total plasma protein level as a stable index of condition (Ots *et al.*, 1998; Alonso-Alvarez *et al.*, 2002; Horak *et al.*, 2002).

Immunocompetence estimation

As an estimate of immunocompetence, ducks were immunized with sheep red blood cells (SRBC, Haemosan, Vienna, Austria), a standard immunological technique that is used to invoke a T-cell dependent antibody response without any concomitant effects of disease (Hudson & Hay, 1976). This assay has been widely used in poultry breeding, and high responses to SRBC are correlated with antibody responsiveness to a variety of antigens and with resistance to several

important parasites (Parmentier *et al.*, 1996). We immunized 36 drakes, excluding four males that had been immunized previously, to determine when SRBC antibody titres peak. All birds in one aviary were immunized in one session. After collection of a pre-immunization blood sample, animals were injected intra-peritoneally with 0.5 mL of a fresh suspension of 10% SRBC in phosphate buffered saline. One week later (28 and 29 October), when antibody titres reach their maximum (A. Peters, unpubl. data), post-immunization blood samples were collected. Antibody concentrations were determined within 3 weeks after plasma collection in a haemagglutination titration assay, following standard protocol (Hudson & Hay, 1976; Peters, 2000). A 20 μL sample was serially diluted in 20 μL PBS in a 96-well plate and incubated with 20 μL of 1% SRBC for 1 h at room temperature. Titres were expressed as the highest well number at which agglutination occurred, correcting for the occasional ($n = 5$) presence of low levels (titre ≤ 1) of heterologous antibodies. Photographs of all plates were taken for consistency in scoring. SRBC used in the agglutination test were taken from the same sheep blood sample as SRBC used for immunization.

Plasma analysis

Plasma carotenoids were analysed by spectrophotometry (Tella *et al.*, 1998; Bortolotti *et al.*, 2000). To 100–110 μL of acetone, 15–30 μL of plasma was added, and centrifuged at 1500 *g* for 10 min. We determined absorbance of the supernatant at 446 nm using a Palm-SPEC™ spectrophotometer (Ocean Optics, Inc., Eerbeek, Netherlands). Concentration of carotenoids was calculated from the absorption coefficient of lutein in acetone at 446 nm (J.J. Negro pers. comm). Linearity was confirmed in a dilution series (1 : 3 to 1 : 20) of five plasma samples. Total plasma protein was analysed colorimetrically using the Fluitest® total protein kit (Biocon® Diagnostik, Marienhagen, Germany), which follows the Biuret method. We mixed 500 μL of Biuret reagent, and 10 μL of plasma and measured absorbance with the Palm-SPEC™ spectrophotometer at 546 nm against a standard of 60 g L^{-1} bovine albumin.

Bill colour measurement

The mallard bill is perceived by humans as greenish-yellow due to the presence of carotenoids (Lönnberg, 1938). HPLC analysis indicated that the carotenoids present in the mallard duck bill are lutein (the largest fraction) and approximately equal fractions zeaxanthin and 3-de-hydrolutein (S. Andersson and A. Johansson pers. comm). We measured reflectance of the bill from 320 to 700 nm using a S-2000 spectroradiometer with a DH-2000-FHS Deuterium–Halogen light source (Ocean Optics). Inclusion of the ultraviolet (320–400 nm, UV) is necessary as ducks are sensitive to UV light (Parrish *et al.*, 1981). To exclude ambient light and standardize measuring distance a cylindrical plastic tube was mounted on the bifurcated fibre optic probe. The probe was held at a 90° degree angle to the bill, and reflectance was measured on three different, standardized spots (11.3 mm^2) between 5 and 10 mm under the right nostril, always by the same person (A.G.D.). Reflectance (R) was calculated relative to a white standard (WS-2) with the program Spectrawin 5.0 (Avantes). The three spectra obtained for each bird were averaged and mean reflectance was summarized over 3 nm steps ('binned'; Grill & Rush, 2000) before statistical analysis.

The reflectance spectrum is double-peaked, with a prominent trough around 450 nm, the area of peak carotenoid absorption (Fig. 3a). Double-peaked spectra are difficult to describe in terms of hue and chroma (Cuthill *et al.*, 1999) and we mathematically summarized the spectra using principal component analysis (PCA). This method makes no assumptions about how reflectance variation is perceived, (unlike methods optimized for human-like visual systems, for example Endler, 1990) or which aspects of the spectrum might be important (Cuthill *et al.*, 1999). Although brightness variation between spectra is often large and not particularly meaningful (Cuthill *et al.*, 1999) we chose not to correct for brightness by using standardized spectra as we preferred to extract all information from the spectra using only one method. In addition to PCA we calculated UV chroma (R_{UV}/R_{total}; Andersson *et al.*, 1998) and carotenoid chroma [$(R_{450nm} - R_{700nm})/R_{700nm}$; Örnborg, 2002). The former represents relative UV reflectance while the latter represents relative reflectance around peak absorbance of carotenoids and has been shown to accurately predict carotenoid (lutein) content of yellow feathers (Örnborg, 2002).

Principal component analysis was performed including all 40 spectra. However, as PCA depends on all data included in the calculation, the PCs may differ depending on the data set analysed (Cuthill *et al.*, 1999; Grill & Rush, 2000). Therefore, we performed two additional PCAs: one including only the spectra of the 36 males that were used in the immunocompetence test and one including only the spectra of the 15 males for which we had sperm swimming measurements. We repeated all statistical analyses of immunocompetence and sperm quality using the respective alternative sets of PCs. These analyses resulted in the same conclusions, with similar *P*-values, and we therefore present only the results of the analysis based on the PCs calculated for all 40 males.

Sperm collection and quality measurement

During the spring breeding season (April to June), sperm samples were collected from seven males in 2002 and eight (different) males in 2003, including at least one male from each family. Males were housed in

isolation during the entire season. Following the massage procedure described in Lake & Stewart (1978), ejaculates were collected from each male at weekly intervals. We included in our analysis only sperm samples that did not contain visible traces of faeces or urine, thus analysing on average 4.2 (SD = 1.6) ejaculates per male. Immediately after ejaculate collection, sperm was diluted to a final concentration of 3 million cells mL^{-1} in Ringer Lactat and blood-buffer (Ringer-plus-ejaculate: blood-buffer = 10 : 1). For this, we followed the protocol by Froman & Feltmann (2000), with the modification that we used only the clear phase of the blood buffer suspension, as no addition of erythrocytes was needed to keep sperm activity constant (A.G. Denk, pers. obs.). Sperm swimming speed was analysed using a Hobson Sperm Tracker (Hobson Tracking Systems Ltd., Sheffield, England). We injected the diluted sperm suspension in a prewarmed (38 °C) MicroCell swimming chamber (50 micron depth; Conception Technologies, San Diego, CA, USA). Observations were made at 38 °C using a 4× brightfield objective under a pseudo dark-field condition with a Ph3 annular phase ring. For each ejaculate three replicates were recorded over a period of 15 min. Minimum track time was set at 1.2 s. As a measure of sperm quality we used the seasonal average of the straight-line velocity of sperm between 8 and 14 min after addition to the extender. This measure of ejaculate quality determined fertilization success of sperm in a competitive situation (artificial insemination of female mallards with a mixture of sperm from two males; A.G. Denk, A. Holzmann, A. Peters, E.L.M. Vermeirssen & B. Kempenaers, unpubl. data). All sperm quality measurements in both years were performed by the same person (A.G.D.), and measurement repeatability was high ($r_{adj} = 0.8$, $P < 0.001$, repeatabilities calculated according to Lessells & Boag, 1987). Between-ejaculate repeatability was not particularly high ($r_{adj} = 0.12$), similar to what is known from zebra finches (Birkhead & Fletcher, 1995). Part of the variation may have resulted from invisible contamination with faeces or urine, incidental variation in the success of the massage procedure or underlying temporal variation in sperm quality produced by individual males (as observed in other studies, C. Stunden, pers. comm.).

Statistical analysis

Because birds belonged to family groups and were housed in three separate aviaries, the possibility existed that family- or aviary-membership affected male condition, sexual signalling or immune response. Therefore, we initially controlled for potential family- or aviary-based differences by including 'family' and 'aviary' as random effects. As group sizes were different we used restricted (residual) maximum likelihood (REML) regression models. Contribution of fixed and random terms was tested by excluding these from the model and examining the change in deviance, which follows a chi-square distribution. Nonsignificant terms were excluded from the initial, full model one by one, in order of lowest effect size. Lack of contribution of excluded terms was confirmed by including them in the final model, which contained all terms with $P < 0.1$. We present significance details for all terms when excluding them from the final model. Unless mentioned otherwise in the results section and Table and figure legends, no significant contributions of 'family' or 'aviary' were detected in the models (all $P > 0.5$). In the latter case, results of univariate correlations or linear regression models (R^2) are also presented. Data were analysed using Genstat® (Genstat, 2002).

Results

Reflectance spectra

The bill of the male mallard was reflective across a large part of the spectrum, with the exception of the band between 400–500 nm. Such a spectrum with a steep increase in reflectance around 500–550 nm and then a plateau at longer wavelengths is typical for carotenoids, while the small peaks in reflectance in the trough of the spectrum are typical of lutein absorbance (S. Andersson, pers. comm.). PCA indicated that three PCs summarized >99% of spectral variation. Their coefficients reflect the multi-peaked nature of the spectrum, with reflectance at most wavelengths correlated with that at other wavelengths (Fig. 1, for a detailed description how to relate PCs through their coefficients to the original spectra, see Cuthill *et al.*, 1999; Grill & Rush, 2000). As is typical for PCA of nonstandardized reflectance spectra, PC1 summarized most (93%) of the variance between spectra with high and positive loadings across the entire wavelength range (Fig. 1) and basically represents achromatic brightness (correlation with brightness, $r > 0.99$, $P < 0.001$), which often contains little relevant information (Cuthill *et al.*, 1999). The other PCs then describe

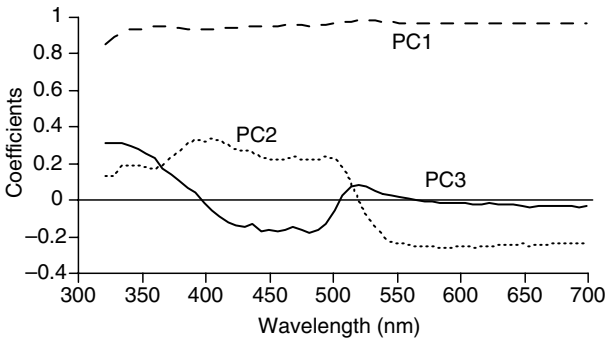

Fig. 1 Coefficients relating the three principal components (PC1-3) to the original reflectance spectra ($n = 40$ males, 21 October 2002; see also Fig. 3a and Materials and methods for details).

spectral shape ('colour'), typically a more informative characteristic of the spectrum (see Cuthill *et al.*, 1999). PC2 (describing approximately 60% of spectral shape variation) showed positive coefficients for shorter wavelengths and negative for wavelengths >500 nm (Fig. 1). In accord with positive PC2 coefficients around 450 nm, the area of peak xanthophyll absorption (Örnborg, 2002), PC2 is highly and negatively ($r = -0.9$, $P < 0.001$) correlated with carotenoid chroma. Coefficients of PC3, describing approximately 40% of variation in spectral shape, were positive for wavelengths in UV and for wavelengths between 500 and 550 nm, while PC3 coefficients were negative for the intermediate wavelengths (Fig. 1). Accordingly, PC3 is positively correlated with UV chroma ($r = 0.5$, $P < 0.001$).

Condition and carotenoids

Results of the linear (mixed) models examining relationships between bill colour principal components and male condition and carotenoids are summarized in Table 1. None of the PCs showed evidence of condition dependence as they were not related to body mass controlling for structural size or plasma protein level. PC1, bill brightness, was not related to plasma carotenoid level. Conversely, PC2 as well as PC3 decreased with increasing plasma carotenoid concentration (Fig. 2; Table 1).

Immunocompetence

Most ducks (28 of 36) produced antibodies (titres: range 0–6, median = 2.5, mean ± SE = 2.3 ± 0.3). Antibody production was not affected by the condition of a male: neither general body condition [Wald $\chi^2_1 = 1.36$, n.s.; body mass with body size (Wald $\chi^2_1 = 0.11$, .n.s.) as forced co-variate] nor plasma protein (Wald $\chi^2_1 = 1.77$, n.s.) significantly predicted antibody titre. Similarly, of the bill colour parameters, neither PC1 (Wald $\chi^2_1 = 2.46$, n.s.) nor PC2 (Wald $\chi^2_1 = 0.02$, n.s.) made a significant contribution to the model of titre. In contrast, PC3 significantly predicted the strength of the immune response to SRBC (Wald $\chi^2_1 = 6.99$, $P < 0.01$). A lower score for PC3 was associated with a higher anti-SRBC titre (Fig. 3a, as corroborated by the negative correlation between PC3 and titre: $r = -0.40$, $n = 36$, $P < 0.05$), suggesting that males with lower relative reflectance in UV were more immunocompetent. Indeed, from the visual representation in Fig. 3b it is confirmed that males that produced higher than average titres had bills that reflected less in UV (particularly below 360 nm) and more in longer wavelengths (above 550 nm).

Table 1 Colour, carotenoids and condition. Depicted are statistical details (Wald-χ^2 and P with d.f. = 1) of three linear (mixed) models examining relationships between bill colour PC1–PC3 and plasma carotenoid level and male condition (plasma protein and body mass controlling for skeletal size).

	PC1		PC2‡		PC3§	
	χ^2	P	χ^2	P	χ^2	P
Carotenoid level*	0.76	0.38	3.91	0.048	6.30	0.012
Protein level	0.93	0.34	0.72	0.40	0.34	0.56
Body condition†	0.32	0.57	0.44	0.51	0.00	0.96

*See Fig. 2.
†Body mass with first PC of body size as forced co-variate (Green, 2001).
‡Mixed model, controlling for a family effect (Wald $\chi^2 = 4.28$, d.f. = 1, $P < 0.05$).
§Mixed model, controlling for a family effect (Wald $\chi^2 = 5.92$, d.f. = 1, $P < 0.05$).

Sperm quality

Average sperm swimming performance was highly variable between males (mean = 44, SD = 11, range 27–61 μm s^{-1}). Neither PC1 (Wald $\chi^2_1 = 0.9$, n.s.) nor PC2 (Wald $\chi^2_1 = 0.6$, n.s.) nor male age (Wald $\chi^2_1 = 0.3$, n.s.) significantly explained any of this variation in sperm straight-line velocity. However, average sperm swimming speed increased significantly with decreasing PC3 (Wald $\chi^2_1 = 6.02$, $P < 0.05$; Fig. 4), controlling for a difference in sperm performance between sampling years (Wald $\chi^2_1 = 3.91$, $P < 0.05$). This relationship also held without controlling for year (PC3 – sperm velocity pair wise correlation $r = -0.53$, $n = 15$, $P < 0.05$). In view of the large variability in sperm velocity within males and the different number of good ejaculates obtained per male, we repeated the analysis including only the fastest ejaculate per male. This resulted in a similar negative relationship between PC3 and sperm velocity (Wald $\chi^2_1 = 4.03$, $P < 0.05$, controlling for a difference in sperm performance between sampling years Wald $\chi^2_1 = 9.11$, $P < 0.01$).

Discussion

Our results show that the mallard bill could signal information on important male traits, and this information was contained in the spectral shape, or the colour, of the bill. To the mallard visual system, the dual peaked spectrum (see Fig. 3b) represents a nonspectral colour, through stimulation of nonspectrally adjacent cones (c/f purple for humans, Burkhardt, 1989). Such dual-peaked spectra are typically difficult to describe in terms of hue and chroma, and we summarized the spectra using PCA (Cuthill *et al.*, 1999). The second PC represents the carotenoid signal, with lower scores indicating higher carotenoid content of the bill and plasma. First, there is a negative correlation between PC2 and plasma carotenoid levels (Fig. 2a). Secondly, PC2 correlates negatively with carotenoid chroma, the colour component that represents carotenoid content of yellow feathers (Örnborg, 2002). Thirdly, PC2 has positive coefficients to reflect-

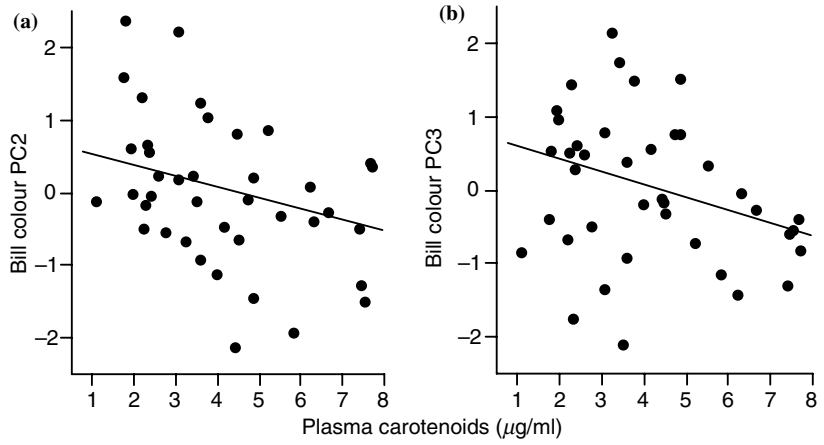

Fig. 2 Relationship of bill colour (a) PC2 and (b) PC3 with circulating plasma carotenoids. Dots show observed values, the lines ($0.68 - 0.15 \times$ carotenoids and $0.79 - 0.20 \times$ carotenoids, respectively) are predicted by linear mixed models (see text and Table 1 for details).

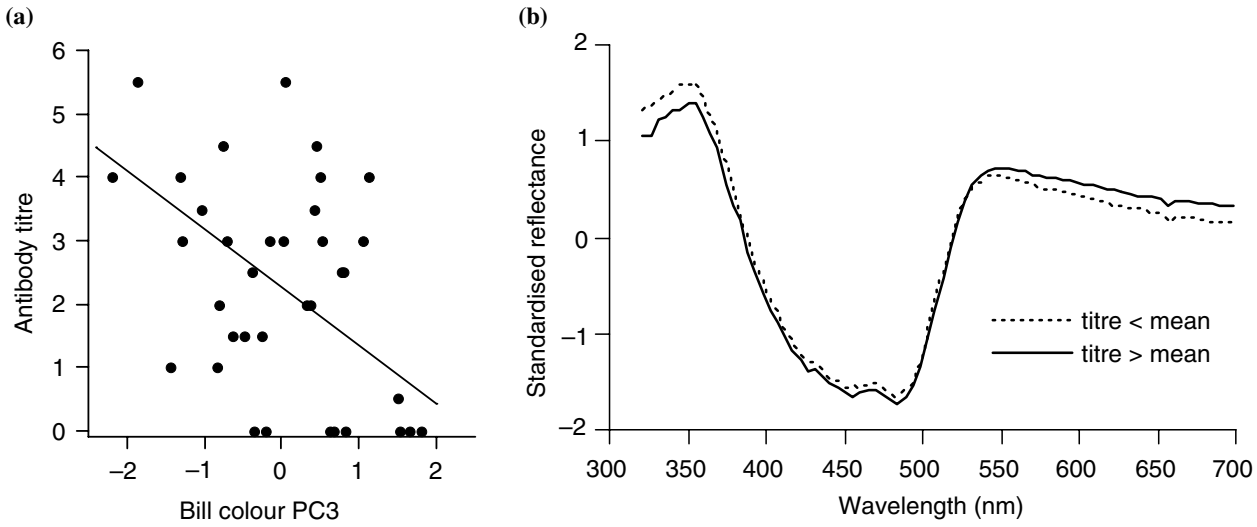

Fig. 3 Immunocompetence and bill colour in mallards. (a) males with lower PC3 scores produce higher antibody titres in response to a single immunization with SRBC. Dots show observed values ($n = 36$), the line ($2.3 - 0.70 \times$ PC3) is predicted by a linear model ($R^2 = 0.16$, see text for details). (b) pre-immunization spectra of the bill of male mallards that produced above-average (solid line, $n = 19$) and below-average (dashed line, $n = 17$) antibodies to a single immunization with SRBC. Spectra are averaged, smoothed and standardized by brightness to allow visual assessment of spectral shape.

ance around 450 nm (Fig. 1), the area of peak absorbance of xanthophylls (Örnborg, 2002). Thus lower PC2 scores should correspond to higher carotenoid content of the bill, in the same way that feather colour reflects its carotenoid content (Örnborg, 2002; Saks *et al.*, 2003a). Positive correlations between ornament colour and plasma carotenoid level have been demonstrated for the red bill in zebra finch males as well as females (McGraw *et al.*, 2003) and the ceres, lores and tarsi in kestrels of both sexes (Bortolotti *et al.*, 1996). Our data suggests that in male mallards, lower PC2 scores of the bill signal higher carotenoid content of the bill as well as plasma.

Likewise, lower PC3 scores were also associated with higher carotenoid levels in plasma (Fig. 2b). However, unlike the situation for PC2, this is unlikely to be directly linked to carotenoid content of the bill as loadings for PC3 for reflectance around 450 nm are small but negative. Rather it seems plausible that the relationship between lower PC3 scores and increasing carotenoids can be interpreted as an increase in bill colour quality mediated by antioxidant effects of circulating carotenoids. Keratin ornaments such as the bill of the duck are sensitive to oxidative stress (von Schantz *et al.*, 1999). Circulating carotenoids play a key role in scavenging free radicals and other reactive molecules, thereby reducing oxidative stress (Bendich, 1993; Mortensen *et al.*, 1997). As carotenoids are destroyed during their antioxidant action, circulating carotenoid levels can reflect oxidative stress levels (von Schantz *et al.*,

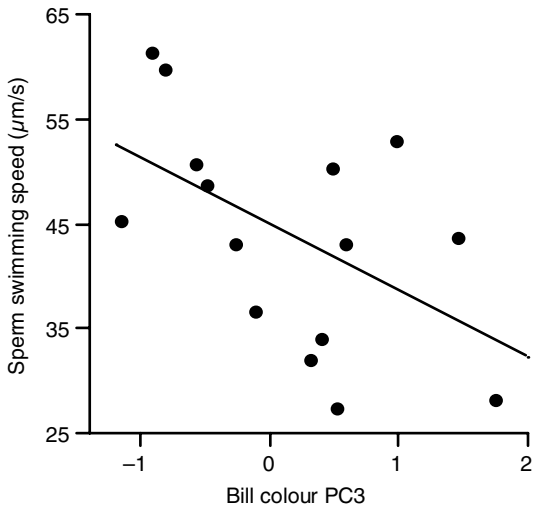

Fig. 4 Sperm swimming performance and bill colour in mallards. Males with lower PC3 scores (measured during the autumn pairing season of 2002) produce faster sperm, with higher straight-line velocity (measured during the breeding seasons of 2002 and 2003). Dots show observed values ($n = 15$), the line ($43.2 - 6.4 \times$ PC3) is predicted by a linear model ($R^2 = 0.46$) controlling for annual variation in sperm performance (see text for details).

1999). If bill colour PC3 is sensitive to oxidative damage, then higher levels of circulating carotenoids provide greater antioxidant protection and consequently lower PC3 scores. If carotenoid availability depends on individual quality (Olson & Owens, 1998; Hill *et al.*, 2002), then protection of ornaments against immune-induced oxidative stress by circulating carotenoids might be a general mechanism enforcing honesty on a variety of (sexual) ornaments (von Schantz *et al.*, 1999), even including those that do not depend on deposited carotenoids.

In accordance with a negative relationship between PC3 and carotenoid reserves and the sensitivity of the immune system to oxidative stress (von Schantz *et al.*, 1999), males with lower PC3 scores produced more antibodies in response to a simulated immune challenge (Fig. 3a). That sexual signals may reflect some underlying quality of the immune system and that females could use these to select an immune-superior mate is a popular notion (Folstad & Karter, 1992; Lozano, 1994; Westneat & Birkhead, 1998; von Schantz *et al.*, 1999). Indeed, several recent studies have shown that ornament elaboration can predict immune responsiveness to a test stimulus (immunization with SRBC or similar antigen): tail-length in barn swallows (*Hirundo rustica*, Saino & Møller, 1996), song characteristics in European starlings (*Sturnus vulgaris*, Duffy & Ball, 2002), spur growth in pheasants (*Phasianus colchicus*, Ohlsson *et al.*, 2002), and carotenoid-based plumage coloration of male greenfinches (*Carduelis chloris*, Saks *et al.*, 2003a), although positive correlations between ornament and immune quality are by no means universal (Saino & Møller, 1996; Birkhead *et al.*, 1998; Faivre *et al.*, 2003b). We demonstrated a strong association between pre-immunization bill colour PC3 and subsequent investment in antibody production in male mallards, indicating that female mallards could use bill colour variation to select a more immune responsive mate.

A more immunocompetent mate might benefit females directly and indirectly. Mallards, unlike 97% of birds, have an intromittent organ (King, 1981). This increases the likelihood of sexually transferred pathogens compared with other birds where sperm is not deposited inside the female reproductive tract (Sheldon, 1993). Such sexually transmitted parasites can have profound negative effects on males as well as females (Sheldon, 1993; Cunningham, 2003, and references therein). As individuals infected with sexually transmitted diseases are unlikely to show outward signs of infection (Sheldon, 1993), females could derive significant direct benefits from selecting a mate with a vigorous immune system. Moreover, more healthy males could contribute to female reproductive success by providing more effective behavioural protection from harassment during foraging (Williams, 1983). Finally, more immunocompetent males might pass on superior resistance genes to their offspring (Hamilton & Zuk, 1982).

In addition to negative relationships with carotenoid reserves and immune responsiveness, lower PC3 scores were associated with greater average seasonal sperm swimming speed (Fig. 4). This aspect of sperm quality is consequential in mallards, as sperm velocity determines fertilization success after artificial insemination with a sperm mix (A.G. Denk, A. Holzmann, A. Peters, E.L.M. Vermeirssen & B. Kempenaers unpubl. data) and sperm velocity, or mobility, has been shown to determine fertilization success in other species (poultry, Birkhead *et al.*, 1999; humans, Holt *et al.*, 1989). The relationship between bill colour and sperm quality is unlikely to represent immediate between-male differences in condition or carotenoid reserves, since we determined bill colour during the mate choice period in autumn and sperm quality during breeding in spring, months earlier or later. Rather, this result suggests that differences in bill coloration represent underlying quality differences between males that persist over seasons or years. That PC3 might reflect male quality is supported by the observation that it varies with family membership (a significant random family effect, Table 1), suggesting genetic components to relative UV reflectance. Persistent male qualities should interest female mallards as they choose mate months before breeding (Williams, 1983), and our results suggest that superior males have lower PC3 during pairing and faster sperm on average during breeding.

Although patterns of male ornamentation and mating success in birds are compatible with more ornamented males having superior fertilizing ability (Sheldon, 1994),

direct evidence for an association between male ornamentation and ejaculate quality is scant, especially for birds. In three species of gazelle, semen quality was negatively related to fluctuating asymmetry of the horns, a trait that may be targeted by female choice (Gomendio *et al.*, 2000). Most evidence that male phenotype can signal fertilizing ability comes from fish, where sperm reserves have been shown to correlate with preferred traits such as sexual display rate (Matthews *et al.*, 1997) and carotenoid coloration (Pitcher & Evans, 2001), but not always positively (Engen & Folstad, 1999; Liljedal *et al.*, 1999). In birds, however, despite some studies suggesting that preferred males may produce superior sperm (Nitchuk & Evans, 1978; Tsuji *et al.*, 1992; Sheldon, 1994), detailed examination revealed no evidence that preferred male phenotypic traits are correlated with the quality of sperm in zebra finches (Birkhead & Fletcher, 1995; Birkhead *et al.*, 1998), pea fowl (Birkhead & Petrie, 1995) and sedge warblers (Birkhead *et al.*, 1997). Perhaps fertilization success of preferred males in mallards might depend more on sperm quality than in other bird species, as a consequence of their intromittent organ. Despite the fact that females show strong preferences for certain males, they appear unable to resist persistent unwanted males and forced extra-pair copulation commonly occur (Cunningham, 2003). Population sex ratios are male-biased, and nonpreferred males remain unpaired, and as all males attempt and succeed in extra-pair copulations (Davis, 2002b), some inseminations result from nonpreferred males (Cunningham, 2003). If a female's own – preferred – mate had sperm with a superior competitive ability this might assist in reducing fertilization success of unwanted forced extra-pair copulations.

Consequences for female choice

Female mallards choose a mate many months before breeding, and although males abandon their mates during incubation and do not contribute to offspring care, males contribute to female reproductive success not only indirectly, but also directly, by protecting their mate from harassment during foraging (Williams, 1983). Females show strong preferences for certain males (for example see Davis, 2002a,b; Cunningham, 2003), and bill colour is a strong predictor of female mate preference (Omland, 1996a). Our study showed that females could obtain information on male oxidative stress levels and immune and sexual competence by examining bill colour, as males with lower PC3 scores had higher carotenoid reserves, greater immune responsiveness and faster swimming sperm. Much of the variation in bill reflectance between males lies in the UV range, around 350 nm, as is confirmed by the comparison of males that produced high and low antibody responses (Fig. 3b). Mallards are not as sensitive to UV wavelengths as other birds, with peak sensitivity of the short wavelength cone around 420 nm (Jane & Bowmaker, 1988). Nonetheless, ducks have demonstrated UV sensitivity since they are highly responsive to light between 340 and 360 nm (Parrish *et al.*, 1981). Moreover, birds with a vision system similar to mallards, black grouse, *Tetrao 0tetrix*, could distinguish a slight difference in reflectance in the UV range (Siitari & Viitala, 2002). The maximum sensitivity of the UV cone of galliform birds - to which grouse belong - also lies around 420 nm as in mallards (Siitari & Viitala, 2002). Thus, mallard females should theoretically be capable of perceiving the UV variation in bill coloration. Although we know that females prefer drakes with 'yellower' bills as mates (Omland, 1996a), colour was assessed by human vision, and we have no information on the role of the UV part of the spectrum. However, when Omland (1996b)) in his experimental study of female mate preferences increased or decreased bill colour with yellow and black markers, both treatments reduced attractiveness, indicating that alteration of the reflectance spectrum in the UV could affect female preferences. Spectrometric analysis of bill reflectance of preferred and nonpreferred males could establish if female mallards indeed prefer males with lower relative UV reflectance, as our study suggests they might, to obtain a more fertile mate with a more active immune system that suffers less from oxidative stress.

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