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Individual quality and carotenoid-based plumage ornaments in male red bishops (*Euplectes orix*): plumage is not all that counts

ALICE U. EDLER and THOMAS W. P. FRIEDL*

Animal Physiology and Behaviour Group, Institute for Biology and Environmental Sciences, Carl von Ossietzky University Oldenburg, PO Box 2503, 26111 Oldenburg, Germany

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Males in many bird species develop elaborate carotenoid-based plumage ornaments that play an important role as signals of individual quality in intra- or intersexual selection. In the present study, we investigated which of several factors related to male condition and health affect the brightness and coloration of the carotenoid-based orange–red breeding plumage in males of the red bishop (*Euplectes orix*), a polygynous and sexually dimorphic weaverbird species. The study revealed a very complex pattern, with the relationships between plumage traits and both heterophil-to-lymphocyte ratio and blood parasite load varying considerably among seasons, suggesting a strong influence of environmental conditions. Furthermore, overall condition of males strongly affected the association pattern between plumage traits and other factors, with males in bad condition being forced to allocate resources away from plumage elaboration to body maintenance or the enhancement of immune functions, whereas males in good condition can afford to invest in plumage ornamentation without obvious detrimental effects on health. Thus, females cannot rely on plumage characteristics alone to gather information on male quality, but have to assess additional traits that advertise general male health status. Perhaps surprisingly, testosterone levels were not related to male plumage characteristics. *Biological Journal of the Linnean Society*, 2010, **99**, 384–397.

ADDITIONAL KEYWORDS: blood parasites – brightness – coloration – condition – health – immunocompetence – physiological stress – plumage – signals – testosterone – weaverbird.

INTRODUCTION

Communication via visual signals has been shown to be important for conveying information not only intrasexually, but also intersexually. Carotenoid-based ornamentation, expressed in brilliant yellows, oranges, and reds, has received much attention, especially in bird species. The coloration of diverse ornaments and appendages, such as wattles (Ohlsson *et al.*, 2003; Smith *et al.*, 2007), combs (Mougeot, 2008), eye-rings (Kristiansen *et al.*, 2006; Pérez-Rodríguez & Viñuela, 2008), and feathers (Karu, Saks & Hõrak, 2007; Peters *et al.*, 2008) is achieved by the deposition of carotenoid pigments into the respective appendages. These pigments cannot be synthesized by vertebrates and therefore need to be ingested as part of the diet and metabolized accordingly. Because of their chemical and physiological properties, carotenoids are not only used in the context of visual signalling, but also function as free-radical scavengers and act as enhancers for the immune system (Krinsky, 1994; Møller *et al.*, 2000; McGraw, 2005).

Carotenoid-based plumage is a common trait in males of many bird species and several studies have shown that the expression of this trait is influenced by a variety of factors, such as testosterone levels (Gonzalez *et al.*, 2001; Duckworth, Mendonca & Hill, 2004), the degree of parasitization (Hõrak *et al.*, 2001; Hill & Farmer, 2005), health (Lozano, 1994; Faivre *et al.*, 2003), and age (Ferns & Hinsley, 2008). For this reason, carotenoid-based plumage plays a vital role in

*Correspondence author.
E-mail: thomas.friedl@uni-oldenburg.de

communication not only on an intersexual level, where females can use visual cues to assess different aspects of quality of a potential mate (von Schantz *et al.*, 1999; Senar & Escobar, 2002; Ferns & Hinsley, 2008; for a review, see Hill, 2002), but also on an intrasexual level, enabling males to assess the individual competitive ability of prospective rivals (Pryke *et al.*, 2002; Pryke & Griffith, 2006; Griggio *et al.*, 2007).

Several hypotheses have linked different aspects of individual quality (i.e. body condition, testosterone levels, parasite load, immunocompetence) to the expression of coloration. The first one was the Hamilton–Zuk hypothesis (Hamilton & Zuk, 1982), which states that plumage coloration functions as an honest indicator of parasite resistance, with bright males having fewer parasites than less colourful individuals (Hamilton & Poulin, 1997; Lindström & Lundström, 2000). This would allow plumage coloration to be used as a reliable visual signal for females when choosing a mate because they would indirectly benefit from mating with such an elaborately coloured male as a result of the increased survival probability of their young through enhanced parasite resistance.

The realization that testosterone might mediate the mechanism for the distribution of energy between immune defence and ornamentation lead to the formulation of the immunocompetence handicap hypothesis (ICHH; Folstad & Karter, 1992; Wedekind & Folstad, 1994), which proposes a link between plumage coloration and different aspects of individual quality. The hypothesis is based on the assumptions that increased testosterone levels lead to immunosuppression and that testosterone enhances male sexual behaviour such as courtship or aggression against male competitors, as well as the expression of sexual ornaments. Given that ornamentation and immunocompetence compete for the same limited resources such as energy and carotenoids, only high-quality males can divert valuable immune enhancing carotenoids away from the immune system and invest in ornamentation without serious risks to their general health (Westneat & Birkhead, 1998; Aguilera & Amat, 2007). Although a link between immunocompetence and plumage coloration has been demonstrated in some experimental studies in zebra finches (*Taeniopygia guttata*; Blount *et al.*, 2003; McGraw & Ardia, 2003), the precise mechanism behind this potential trade-off in the allocation of carotenoids between elaborate ornaments and the immune system is still a matter of debate (Hõrak *et al.*, 2007; Alonso-Alvarez *et al.*, 2008). In addition, the assumption of the ICHH that testosterone acts as an immunosuppressive agent still remains equivocal (Roberts, Buchanan & Evans, 2004).

The intensity of coloration can further be affected by body condition, as demonstrated by Estep, Shawkey & Hill (2006) in red fodies (*Foudia madagascariensis*), where males in better condition displayed brighter feathers. Similar results were found in male cardinals (*Cardinalis cardinalis*; Jawor & Breitwisch, 2004), with red bill colour being a positive predictor of current body condition.

The general aim of the present study was to investigate how much information is conveyed by the orange–red breeding plumage in free-living male red bishops (*Euplectes orix*), a colonial breeding and highly polygynous weaverbird species of sub-Saharan Africa. To achieve this goal, we investigated which of the above mentioned factors (blood parasite infections, testosterone levels, immunocompetence, body condition) affect the expression of plumage coloration in this species. We captured male red bishops over three consecutive breeding seasons, measured individual plumage reflectance, and took blood samples for later analysis of testosterone levels, immunocompetence, and parasitaemia [assessed by the quantitative real-time polymerase chain reaction (PCR)]. Most studies on plumage coloration in birds only analysed the relationship of plumage characteristics with one or two of the factors mentioned above, or investigated variation of plumage characteristics over one study season only. By contrast, in the present study, we perform an analysis in which we assess the effects of several factors together, including interactions, over three study seasons. Thereby we want to obtain a more complete picture of factors affecting carotenoid-based plumage coloration to evaluate when and under what circumstances plumage characteristics indeed might reflect individual quality.

MATERIAL AND METHODS

Study species and area

The red bishop (*E. orix*) belongs to the family of weaverbirds (*Ploceidae*) and occurs in sub-Saharan Africa. It is a sexually dimorphic species, with mature males (aged 2 years and older) showing a brilliant red and black breeding plumage during the breeding season. Red bishops are fairly abundant colonial breeders, occurring along rivers and dams. Males vigorously defend small territories in reed beds consisting mainly of bulrush (*Typha capensis*) and the common reed (*Phragmites spec.*). In their territory, males build as many nests as possible during their tenure, with the number of nests that are built comprising an important determinant of male mating success (Friedl & Klump, 1999, 2000; Lawes, Slotow & Andersson, 2002; Metz, Klump & Friedl, 2009). When a female enters the territory, the male carries

out various courtship displays until the female either leaves the territory or accepts a nest and allows copulation. The main cause of breeding failure (> 70% in each season) is predation by water mongoose, *Atilax paludinosis*, cape cobra, *Naja nivea*, and boomslang, *Dispholidus typus* (Friedl, 2004a; A. U. Edler, unpubl. data). More details on red bishop breeding behaviour are provided in Craig (1974) and Friedl (2004b).

The study took place during three consecutive breeding seasons between 2005 and 2008, with each breeding season lasting from approximately October to March. The study site was a small dam surrounded by bulrush and common reeds, situated in the Addo Elephant National Park, in the Eastern Cape Province, South Africa (33°26′S, 25°45′E).

Field methods

Behavioural observations and the identification of birds via individual colour ring combinations took place every day. Adult birds were caught with mist nets and walk-in traps, which were situated along the edge of the colony. Upon capture, tarsus and wing length were measured and the weight recorded. The body condition index was calculated using the residuals from the regression of body weight on tarsus length and pooled over all three seasons. Data on body condition index were available for a total of 79 males, with 13 of these males having been caught more than once during the course of the study. Blood samples were obtained by puncturing the brachial wing vein with a sterile needle, collecting the blood with a heparinisized microcapillary tube (approximately 20–60 μL), and transferring the sample into a 1.5-mL reaction tube. For blood smears, a drop of blood was placed on a microscope slide and smeared, air-dried and stored in boxes for later analysis. Serum samples were obtained by centrifuging the reaction tubes containing the blood samples with a micro centrifuge (Capsule HF-120; Tomy Kogyo Co., Ltd), removing the upper layer with the serum using glass pipettes and transferring it into 0.5-mL PCR tubes, which were then stored in a freezer. The blood cells remaining in the 1.5-mL reaction tubes were topped with phosphate-buffered saline and refrigerated. In total, we collected blood smears and serum samples from 64 individual males, with ten males being sampled at least twice over the course of this study.

Furthermore, we determined a standardized 'date of capture during season' for all three seasons. This was necessary because there were heavy rains during the winter preceding the second field season, which resulted in breeding starting approximately 1 month earlier than normal. It was therefore not possible to determine an exact start of the breeding season for that year. We defined 'day 0' for each season by determining the hatching date of the first nestling in the colony and subtracting 30 days. Individual 'date of capture' was then calculated from that date. Finally, we *Z*-transformed 'day of capture' to account for differences in length among the three breeding seasons.

Plumage reflectance

Spectral reflectance was measured using an Ocean Optics USB2000 spectrometer (Ocean Optics, Inc.), a GetLight-Hal-S tungsten halogen light source (Getspec; http://www.getspec.com), and a fibre optic reflectance probe, which was fitted with a custom-made black sheath, to exclude ambient light and standardize the distance between probe tip and feather surface. The probe was held perpendicular to the sample and three consecutive scans were taken from approximately the centre of each measured area (throat, head, and lower back; these being the areas in male red bishops showing carotenoid-based plumage coloration), between which the probe was removed and replaced. Reflectance was measured using OOIBase32 spectral analysis software (< 1 nm resolution; Ocean Optics, Inc.) and in relation to a white (WS-2 white reflective tile, 98% reflectance; Getspec), as well as a dark standard (self-made 'dark-box'). Because red bishops do not reflect in the ultraviolet range of 320–400 nm (A. U. Edler & T. W. P. Friedl, unpubl. data), we used the data obtained in the range from 400–700 nm for further analysis. Overall, we were able to collect reflectance data from 52 different individual males, with four of these males having been recaptured at least once over the three study seasons.

To verify that the orange–red breeding plumage in red bishop males is based on carotenoid pigments, we performed the simple two-step chemical extraction method of pigments following McGraw *et al.* (2005). After extraction of feather pigments with heated acidified pyridine and adding a 1 : 1 solution of hexane and tert-butyl methyl ether, the upper organic phase had an orange colour, confirming that the orange–red feather coloration of red bishops is indeed caused by carotenoid pigmentation.

Spectral analysis

The orange–red breeding plumage of red bishop males shows a reflectance spectrum typical for carotenoid-based plumage traits, with no reflectance at the shorter wavelengths and high reflectance at the higher wavelengths corresponding to the orange–red part of the spectrum (Fig. 1A). The collected reflectance data was analysed using a principal component

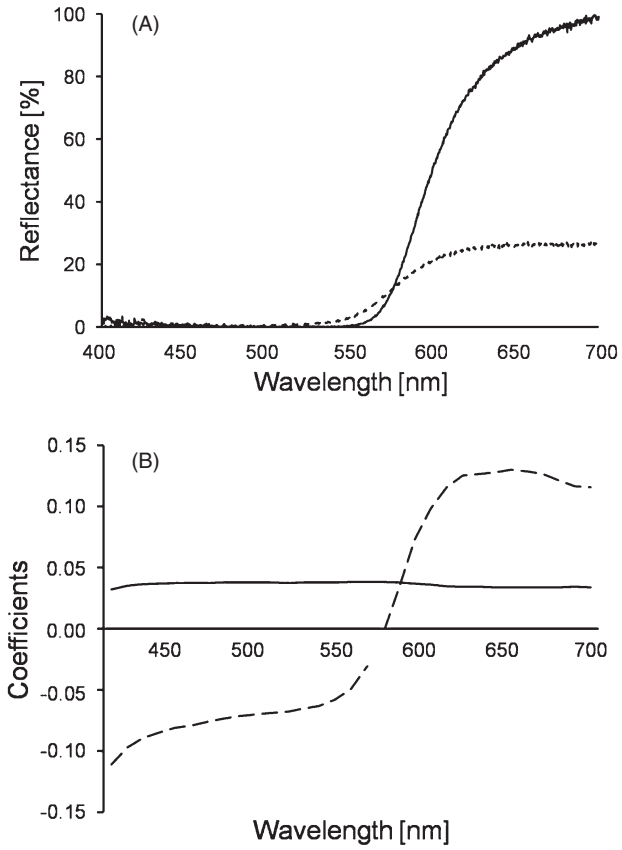

Figure 1. (A) Exemplary reflectance spectra for males with bright (solid line) and dull (dashed line) plumage and (B) principal component (PC) coefficients in relation to wavelength [nm] for PC1 and PC2. The first principal component (solid line) accounts for 83.8% of all variation, and the second (dashed line) accounts for 13.3%.

analysis (PCA; Cuthill *et al.*, 1999; Grill & Rush, 2000; Maney *et al.*, 2008) because this allows a significant reduction of data into principal components (PCs). In comparison with the calculation of colour variables (brightness, chroma, and hue), these PCs are statistically independent from one another and are able to accurately describe complex variation across the examined spectrum (Montgomerie, 2006).

Because we have three measurements from each of the three body regions measured per male, we first calculated an average of the raw data over the three reflectance spectra, divided these into 30 bins spanning 10 nm each (e.g. 400–409 nm, 410–419 nm, etc.) using the median values from each segment and performed a PCA. We obtained two PCs for each of the three body regions measured, with at least 91% variation being explained by two PCs in all three cases. Because both the first PCs and the second PCs obtained for the three body regions correlated highly significantly with each other (Pearson correlation; all $r > 0.4$, all $P < 0.005$), we further collapsed our data by averaging the three sets of 30 bins per individual and performed another PCA. This resulted in the final two PCs explaining 97% of the variation (PC1: 83.8%; PC2: 13.3%).

Based on the obtained loadings, PC1 is interpreted as brightness of the measured sample because these loadings are strong and consistently positive over the entire examined range (Cuthill *et al.*, 1999; Grill & Rush, 2000). PC2 describes the variation in the relationship between reflectances at high and low wavelengths and therefore depicts chroma and hue (Fig. 1B).

Leukocyte profiles

For analysis, the obtained blood smears were stained with Wright's stain and examined under a microscope (Zeiss Axioskop 2 *mot plus*; Zeiss) using a ×630 magnification. We counted the amount of erythrocytes and leukocytes, with the latter being divided into lymphocytes and heterophilic, eosinophilic, and basophilic granulocytes. Consecutive neighbouring microscopic fields were examined, until a total of 100 leukocytes was reached.

The white blood cell count of any given individual can be influenced by several stressors, such as malnutrition, infections, and parasites. Lymphocytes are usually the most common white blood cells in avian haemograms and have a tendency to decrease with rising stress levels. Heterophils, on the other hand, comprising the primary phagocytic white blood cell, increase their circulation in response to infections and stress (Campbell, 1995; Feldman, Zinkl & Jain, 2000; Davis, Maney & Maerz, 2008). The interplay of lymphocytes and heterophils with rising stress and inflammation levels allows the calculation of the heterophil/lymphocyte ratio (HLR), which has been shown to be positively associated with heat-shock protein and glucocorticoid levels (Moreno *et al.*, 2002; Davis *et al.*, 2008) and can thus be considered as a reliable indicator of physiological stress, induced, for example, through chronic stressors or changes in the environment or social rank (Gross & Siegel, 1983; Ots & Hõrak, 1996; Ilmonen *et al.*, 2003; López *et al.*, 2005; Davis *et al.*, 2008). Because stress is known to suppress immune functions (Sapolsky, 1992; Apanius, 1998; Morales *et al.*, 2006), a high HLR, which is indicative of high physiological stress levels, will be associated with a concomitant suppression of immunological condition and an increase in susceptibility to infections or parasitization (Siegel, 1995; Ots, Murumägi & Hõrak, 1998). In light of the complexity of the immune system, and given the fact that the exact mechanisms by which different components of the immune system interact are largely unknown,

assessing overall immunocompetence using a single measure could be inappropriate. Therefore, we mostly interpret and discuss HLR as a measure for physiological stress rather than as an indicator of overall immunocompetence. However, given that HLR reflects physiological stress levels, and that physiological stress is known to suppress at least some components of the immune system (see above), we consider that it is justified to argue that high HLR values indicate not only high physiological stress levels, but also some suppression of parts of the immune system.

To assess repeatability of the counts, ten smears were chosen at random and recounted without knowledge of the previous counts. Using the method described by Lessels & Boag (1987), repeatability measures were high for all counts (lymphocytes: $R = 0.8$, $F_{19} = 42.152$, $P < 0.001$; heterophils: $R = 0.9$, $F_{19} = 89.763$, $P < 0.001$, HLR: $R = 0.9$, $F_{19} = 58.458$, $P < 0.001$).

Testosterone levels

Testosterone concentrations of samples collected in the breeding season 2005/06 were determined at the Max-Planck-Institute for Ornithology with a radioimmunoassay as described in Goymann *et al.* (2006). Plasma samples (mean ± SD volume: 41.5 ± 20.5 μL) were extracted twice with dichloromethane, resulting in an average extraction recovery of 86.0 ± 3.0% (mean ± SD) for ^{3}H-testosterone added prior to extraction. The lower detection limit of the assay was 73 pg per tube, resulting in lower detection limits in the range 39–169 pg mL^{-1} (depending on the amount of plasma available and the respective extraction recovery). To minimize variation, all samples were analysed in one assay with an intra-assay variation coefficient of 4.5%. Because the testosterone antibody (Esoterix Endocrinology) showed significant cross reactions with 5α-dihydrotestosterone (44%), our testosterone measurements may include a proportion of this other androgen.

Testosterone concentrations of samples collected in the breeding seasons 2006/07 and 2007/08 were determined at the Leibniz Institute for Zoo and Wildlife Research (Berlin) using an enzyme immunoassay with a double-antibody technique. First, 0.01–0.05 mL of serum was extracted with 2 mL of butyl *t*-methyl ether : petroleum ether (30 : 70, v/v) for 30 min. The samples were then frozen and the fluid petroleum ether phase was removed and evaporated at 55 °C. The steroids were dissolved in 0.1 mL of 40% (v/v) methanol, and duplicates of 20 μL each were analysed. The assay used a polyclonal antibody raised in rabbits against testosterone-11-hemisuccinate-bovine serum albumin, and the label was testosterone-3-carboxymethyl-oxime-horse radish peroxidase. The testosterone standard curve ranged from 0.4–50.0 pg per 20 μL, and the cross-reactivity with testosterone was 100%, (5α-dihydrotestosterone, 10%; androstenedione, 2%; oestradiol, 0.1%; progesterone, 0.1%). Serial dilutions of a plasma pool from red bishops gave parallelism to the standard testosterone with no differences in slopes ($P > 0.05$). The intra- and inter-assay coefficients of variation were 8.9% and 12.3%, respectively. All results are given in nanograms of testosterone per millilitre of serum.

Parasites

Inferences on the association between parasite loads and the expression of sexual signals such as plumage traits based on data for just a single parasite species might lead to inaccurate interpretations. We therefore decided to develop a method allowing us to quantify infection levels of blood parasites of both the genus *Plasmodium* and *Haemoproteus* because these are the blood parasites most often reported to affect host fitness. Because neither *Leucocytozoon* nor *Trypanosoma* have been reported to parasitize red bishops (Bennett *et al.*, 1992; Edler, Klump & Friedl, 2004), we consider that by quantifying the combined infection levels with *Plasmodium* and/or *Haemoproteus*, we have assessed all the blood parasite species most likely to affect health and/or quality in red bishops.

We determined the intensity of infections with blood parasites of the genus *Plasmodium* and/or *Haemoproteus* by quantitative real-time PCR. In short, parasitaemia (i.e. the number of blood parasites per 100 blood cells) was assessed by amplification of a specific 85-bp fragment within the plastid-like large subunit ribosomal-RNA (LSU-rRNA) gene (Tan *et al.*, 1997), which is conservative across a range of *Plasmodium* and *Haemoproteus* species. By measuring the accumulation of the product during the PCR (in real-time) using a fluorescent-labelled oligonucleotide probe, a threshold was determined at which the fluorescence of the product was raised above background level. The starting quantity of blood parasites in the investigated blood samples of male red bishops was then calculated compared to thresholds determined for standards of known quantity (clones of a 594-bp fragment within the LSU-rRNA gene from *Plasmodium falciparum* including the target sequence) in the same PCR reaction. Given that we used a target sequence that is specific for both *Plasmodium* and *Haemoproteus*, we cannot distinguish between the two blood parasites here. Further details are available upon request.

We were able to successfully quantify blood parasite loads for 48 of the 52 males for which we had plumage

data. We defined males as being parasitized if the number of blood parasites per 100 blood cells was higher than 0.1 because, in passerine birds, very low levels of parasitaemia with *Plasmodium* and/or *Haemoproteus* are generally regarded as being harmless to the hosts (Campbell, 1995). According to this criterion, 31 of the 48 males were not infected and 17 males were infected (i.e. had more than 0.1 blood parasites per 100 blood cells).

Statistical analysis

We used general linear models to test the influence of several factors (season, day of capture, testosterone, HLR, level of parasitization, and body condition) on the expression of plumage coloration (PC1 and PC2) in male red bishops. We visually checked the residual plots and found no indication for deviations from normality or homogeneity of variance. All 52 males for which reflectance data were available were included in the analyses. Four of these males were caught more than once over the course of the study period, and we randomly selected one data set for each of these males to assure statistical independency. We first included all main effects and two-way interactions in the model, and then removed, in a stepwise procedure, all nonsignificant two-way interactions until only significant two-way interactions and the main effects remained in the final model.

All statistical tests were performed using SPSS, version 15.0 or 17.0 (SPSS Inc.), and all given significances are two-tailed.

RESULTS

Influences on plumage coloration

Significant main effects on PC1 were found for season as well as for body condition and HLR (Table 1). Male plumage brightness (indicated by high PC1 scores) was highest in season 2005/06 (mean ± SE: 0.29 ± 0.46), marginally lower in season 2006/07 (0.15 ± 0.13), and by far lowest in season 2007/08 (–0.46 ± 0.11; post-hoc comparisons after Sidak; 2007/08–2005/06, $P = 0.071$; 2007/08–2006/07, $P = 0.046$). Overall, brighter males (having high PC1 scores) were in better condition and had lower HLR values compared to duller individuals.

The only significant main effects on PC2 were found for HLR and the intensity of infections with blood parasites, with redder males having lower HLR values but higher parasite loads than males less saturated in colour. No other significant main effects of the variables investigated on PC1 or PC2 were found (Table 1).

Table 1. Results of general linear model analyses with principal component (PC)1 and PC2 as dependent variables

	PC1				PC2			
	Estimate	*F*	*P*	Partial η^2	Estimate	*F*	*P*	Partial η^2
Season	1.155	5.239	0.011	0.253	1.218	1.794	0.182	0.098
	0.592				0.668			
	0.000				0.000			
Day	–0.033	0.257	0.615	0.008	–0.216	3.358	0.076	0.092
Testosterone	0.010	0.005	0.945	0.000	–0.240	0.903	0.349	0.027
BCI	0.287	7.337	0.011	0.191	0.189	2.425	0.129	0.068
HLR	–1.963	9.718	0.004	0.239	–3.353	8.228	0.007	0.200
Parasites	0.673	3.813	0.060	0.110	1.340	8.371	0.007	0.202
Season × HLR	–0.901	7.873	0.002	0.337	–0.307	3.400	0.045	0.171
	2.030				2.789			
	0.000				0.000			
Season × Parasites	–0.446	7.793	0.002	0.335	–1.082	10.017	< 0.001	0.378
	–1.256				–2.099			
	0.000				0.000			
Day × BCI	–0.180	6.100	0.019	0.164				
Testosterone × HLR	0.714	4.279	0.047	0.121	1.255	4.475	0.042	0.119
BCI × HLR	–0.979	19.547	< 0.001	0.387				
BCI × Parasites	–0.378	7.826	0.009	0.202	–0.489	4.188	0.049	0.113

The final models shown include all the main effects and all significant two-way interactions (see text). Adjusted R^2 was 0.644 for the analysis of PC1 and 0.486 for the analysis of PC2.
BCI, body condition index; HLR, heterophil/lymphocyte ratio.

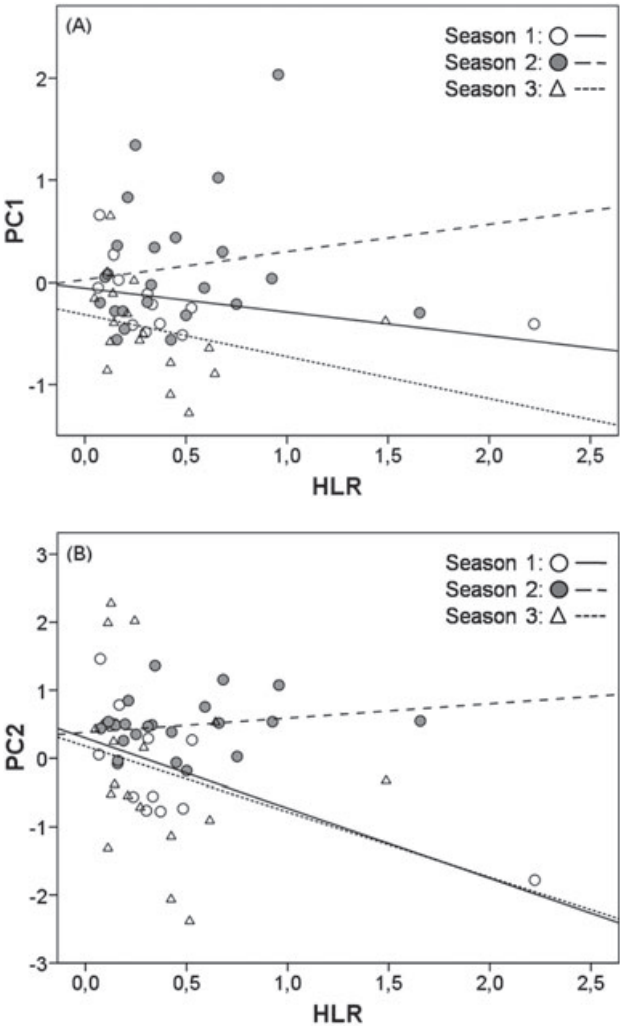

Figure 2. Relationship between heterophil/lymphocyte ratio (HLR) and (A) principal component (PC) 1 and (B) PC2 for each of the three study seasons.

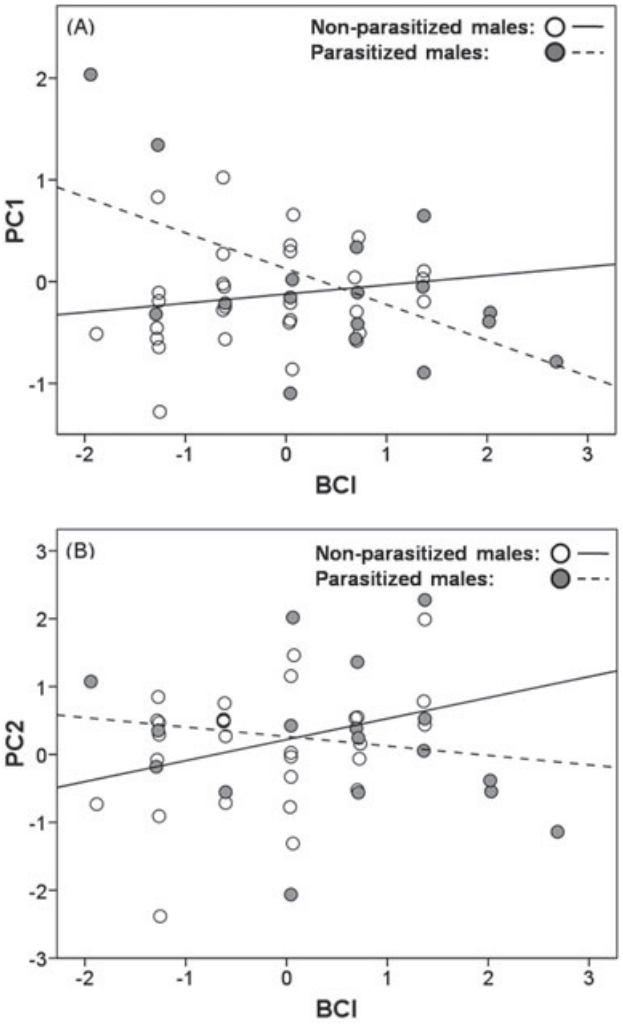

Figure 3. Relationship between body condition (BCI) and (A) principal component (PC) 1 and (B) PC2, for nonparasitized males (less than 0.1 blood parasites per 100 blood cells; empty dots and solid line) and parasitized males (more than 0.1 blood parasites per 100 blood cells; filled dots and dashed line).

There was a significant effect of the interaction between season and HLR on both PC1 and PC2 (Table 1). Both PCs showed a positive relationship with HLR in season 2006/07, whereas the relationships were negative in seasons 2005/06 and 2007/08 (PC1: Fig. 2A; PC2: Fig. 2B). Furthermore, there was also a significant effect of the interaction between season and parasite load on both PC1 and PC2 (Table 1), with no clear or slightly negative relationships between the two PCs and the intensity of infections with blood parasites in seasons 2005/06 and 2006/07, and positive relationships between the two PCs and the intensity of infections with blood parasites in season 2007/08.

The interaction between body condition index and parasite load was significant for both PC1 and PC2, as well as the interaction between testosterone levels and HLR. For males with less than 0.1 blood parasites per 100 blood cells (set to 0; see Material and methods), there was no clear relationship between body condition index and PC1 and a positive relationship between body condition index and PC2, whereas, for males with a parasite load higher than 0.1 blood parasites per 100 blood cells, there was a negative relationship between body condition index and both PC1 and PC2 (PC1: Fig. 3A; PC2: Fig. 3B). For males that suffered from high stress levels (indicated by HLR values above the median), there were negative relationships between testosterone levels and both PC1 and PC2, whereas, for males that did not suffer from high stress levels (indicated by HLR values

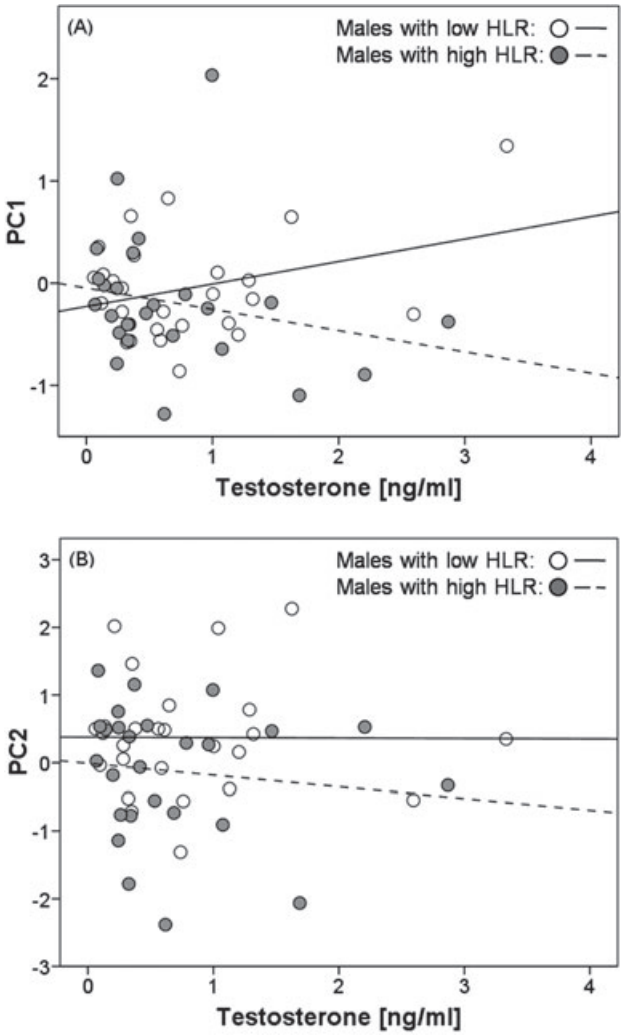

Figure 4. Relationship between testosterone and (A) principal component (PC) 1 and (B) PC2 for males with low (below median; empty dots and solid line) and high (above median; filled dots and dashed line) heterophil/lymphocyte ratio (HLR) values.

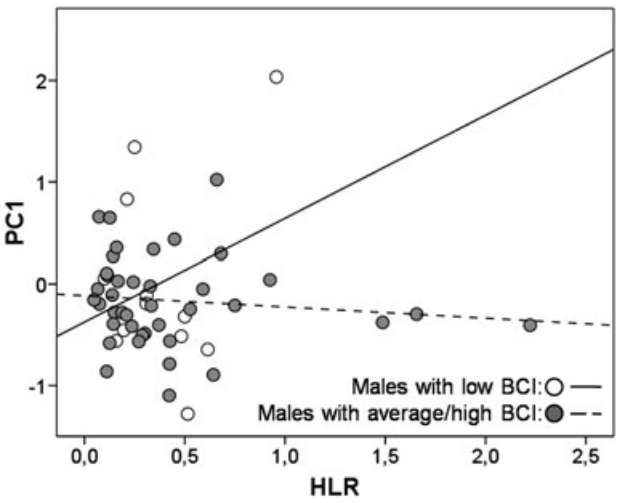

Figure 5. Relationship between principal component (PC) 1 and heterophil/lymphocyte ratio (HLR) for males with a low body condition index (BCI) (lowest quartile of the distribution; empty dots and solid line) and an average to high BCI (upper three quartiles of the distribution; filled dots and dashed line).

below the median), there were positive or no relationships between testosterone levels and both PC1 and PC2 (PC1: Fig. 4A; PC2: Fig. 4B).

PC1 was further influenced by the interactions between day of capture and body condition, as well as by the interaction between HLR and body condition. For males in bad body condition, PC1 decreased over the course of the season, whereas there was no seasonal effect on PC1 for males in good body condition. Finally, for males in very bad body condition (lowest quartile of the distribution), there was a positive relationship between PC1 and HLR (i.e. stress level), whereas, for males in average or good body condition (upper three quartiles of the distribution), PC1 decreased slightly with increasing HLR (Fig. 5).

DISCUSSION

The present study aimed to investigate whether and how the carotenoid-based orange-to-red breeding plumage of male red bishops is influenced by blood parasite loads, testosterone levels, physiological stress levels, and body condition. On the basis of the associations found, we wanted to assess whether plumage brightness (as reflected by PC1) and/or plumage colour (chroma and hue, referred to as 'colour' hereafter; as reflected by PC2) are suitable for use as reliable indicators of different aspects of male quality.

Overall, our analyses revealed a rather complex and complicated picture. We found significant main effects of HLR (as an indicator of physiological stress levels), parasitaemia, and body condition on PC1 and/or PC2, whereas there was no significant main effect of testosterone. In addition, our analyses revealed several significant interaction effects. Below, we will discuss the results obtained in detail and pay special attention to the question of whether plumage characteristics are reliable indicators of male quality as proposed by several hypotheses, including the Hamilton–Zuk hypothesis and the ICHH.

Variation in brightness of plumage patches is most often a result of variation in feather structure, whereas variation in chroma and/or hue is mainly attributable to variation in pigment concentrations (e.g. Shawkey & Hill, 2005; Shawkey *et al.*, 2006). Especially carotenoids that are responsible for most yellow, orange, or red plumage patches in birds play

a specific role because not only are they important for plumage coloration, but also they function as enhancers for the immune system (see Introduction). This suggests that the factors regulating plumage coloration are at least partly different from the factors responsible for variation in plumage brightness. In addition, the relationship between brightness and chroma in carotenoid-based colour patches can be dynamic (Andersson & Prager, 2006). Hence, brightness and coloration may signal different aspects of male quality (Bitton & Dawson, 2008), and we will therefore discuss plumage coloration (as reflected by PC2) and plumage brightness (as reflected by PC1) separately below, even if some of the results obtained were similar for plumage coloration and plumage brightness. Furthermore, given that it is generally not meaningful to discuss the significant main effects of a variable in the presence of significant interactions (Zar, 1999), we mainly focus on the interaction effects when discussing the influence of a variable that shows both a significant main effect and significant interactions in our models.

Plumage coloration

Plumage coloration in red bishop males was affected by HLR (physiological stress levels), with the effect of HLR on plumage coloration differing among the three study seasons, as shown by the significant interaction term between HLR and season (Fig. 2B, Table 1). In both seasons 2005/06 and 2007/08, plumage coloration was negatively affected by high physiological stress levels (high HLR values) and the concomitant suppression of some component of the immune system. These results fit to the proposed trade-off between immune function and carotenoid-based plumage coloration because only males that do not suffer from stress and are therefore in good immunological condition should be able to invest carotenoids in elaboration of plumage coloration rather than in enhancing the status of the immune system. These results are also in accordance with several previous studies demonstrating that more colourful birds are more immunocompetent (Figuerola *et al.*, 1999; Brawner, Hill & Sundermann, 2000; Navara & Hill, 2003; Saks, Ots & Hõrak, 2003), indicating that carotenoid-based plumage coloration can be used as a signal of quality. However, in season 2006/07, there was a different pattern, with plumage coloration (PC2 scores) slightly increasing at higher stress levels (Fig. 2B). This result might be the result of the breeding season in 2006/07 differing significantly in timing from the other two study seasons. Because of unusual and extremely heavy winter rains, the 2006/07 season began approximately 2 months earlier than normal. Because rainfall is known to affect the breeding behaviour of red bishops (Friedl, 2002), these differences in rainfall pattern might explain the reverse relationship between coloration and HLR for that season. For example, the very early start of the breeding season could impose more stress on the males, which are forced to moult earlier and faster into their colourful breeding plumage than usual. Another study on red bishops has already revealed that the relationship between HLR and reproductive performance in male red bishops varied considerably among seasons (Friedl & Edler, 2005), indicating that seasonal variations can indeed have pronounced effects on reproductive parameters.

The effect of parasitaemia on plumage coloration also differed among seasons, with no clear or slightly negative relationships in seasons 2005/06 and 2006/07, and a rather unexpected positive association in season 2007/08 (Table 1). According to both the Hamilton–Zuk hypothesis and the ICHH, a clearly negative relationship between parasitaemia and plumage coloration would be expected because parasites are assumed to depress the expression of carotenoid-based plumage coloration through a trade-off in carotenoid allocation between immune function and its purpose as a colourant. Indeed, such a negative relationship between parasite load and plumage colour has been shown in a variety of bird species, such as yellowhammers (*Emberiza citrinella*; Sundberg, 1995), greenfinches (*Carduelis chloris*; Merilä, Sheldon & Lindström, 1999), house finches (*Carpodacus mexicanus*; Hill & Farmer, 2005), and serins (*Serinus serinus*; Figuerola, Domenech & Senar, 2003). On the basis of the inconsistencies of the relationship between parasitaemia and plumage coloration across seasons as found in the present study, and the lack of a clear negative effect of blood parasite infections on reproductive performance in male red bishops reported in other studies (Edler *et al.*, 2004; A. U. Edler & T. W. P. Friedl, unpubl. data), we suggest that infections with *Plasmodium* and/or *Haemoproteus* have no strong detrimental effects on aspects of male health in red bishops measured in the present study, at least at the low to moderate infection levels found. This appears to be in contrast to the results obtained in other studies that have shown a negative effect of blood parasites on host condition (Ots & Hõrak, 1998; Hatchwell *et al.*, 2000; Dyrcz *et al.*, 2005). However, given that most individuals host a whole parasite community, it is perhaps not surprising that strong negative effects of parasitaemia on the expression of plumage traits are not always detected when only a single parasite or a subset of parasite species are investigated.

The fact that there is a significant interaction between parasite load and body condition on plumage coloration, however, indicates that infections with *Plasmodium* and/or *Haemoproteus* can have some

detrimental effect in red bishops, at least under some circumstances. Whereas, for nonparasitized males (i.e. males with less than 0.1 blood parasites per 100 blood cells), more intensely coloured individuals are in better condition than less colourful ones, plumage coloration decreases with increasing body condition in parasitized males (Fig. 3B). This indicates that there is no trade-off between body condition and plumage coloration for males that do not suffer from detrimental effects caused by blood parasites. By contrast, when there is an additional challenge to the immune system caused by blood parasites, males obviously have to trade-off the allocation of dietary carotenoids to either immune defence or plumage elaboration. In a study on blackbirds using experimentally induced parasite infections, Baeta *et al.* (2008) were able to show that males supplemented with carotenoids were able to maintain bill colour when simultaneously fighting parasites. In comparison, nonsupplemented males displayed reduced bill coloration. This result led to the conclusion that only those individuals with a carotenoid-rich diet are able to handle costly parasite infections, confirming that carotenoids are subjected to an allocation trade-off between immunological responses to parasites and ornament elaboration.

According to the ICHH, testosterone is supposed to enhance male behavioural as well as morphological traits involved in mate acquisition. For example, in bird species where the males have carotenoid-based ornamental traits, males with higher levels of testosterone have been shown to display redder ornamentation (Duckworth *et al.*, 2004; McGraw, Correa & Adkins-Regan, 2006). In the present study, however, we found no significant main effect of testosterone on plumage coloration, although there was a significant effect of the interaction between testosterone and HLR (Table 1). For males that did not suffer from high stress levels (i.e. males that had HLR values below the median of 0.41), there was no clear relationship between testosterone levels and plumage coloration, whereas, for males with high stress levels (i.e. males that had HLR values above the median of 0.41), high testosterone levels were related to reduced plumage coloration (Fig. 4B). This effect might be a consequence of the immunosuppressive character of testosterone (i.e. directly or indirectly), with high hormone levels forcing the individuals to allocate carotenoids away from plumage elaboration to support the suppressed immune system when they suffer from high stress levels and the concomitant increase of susceptibility to infections and parasites.

Plumage brightness

As stated above, there are surprisingly few studies that have investigated the signalling value of plumage brightness, with brightness here referring to spectral intensity (i.e. the total amount of reflectance over the entire wavelength spectrum measured). Unfortunately, the term 'brightness' is often misused in the animal coloration literature, with birds having a more saturated plumage (i.e. higher chroma and/or hue values) described as being 'brighter' than individuals with less saturated plumage coloration (Montgomerie, 2006). Therefore, caution is required when interpreting the results of studies reporting signalling values of plumage characteristics with respect to what component of plumage measurements the authors are referring to.

Overall, many of the results obtained in red bishop males for plumage brightness were similar to the results obtained for plumage coloration above. Similar to plumage coloration, the relationship between HLR and plumage brightness differed among seasons, with the expected negative relationships in seasons 2005/06 and 2007/08 and a positive association in season 2006/07 (Fig. 2A).

Likewise, just as for plumage coloration, the interaction between parasite load and season was highly significant, with no clear or slightly negative relationships between parasite load and plumage brightness in seasons 2005/06 and 2006/07, and a positive association in season 2007/08. In addition, the significant effect of the interaction between parasite load and body condition on plumage brightness was similar to that found for plumage coloration. Whereas, for nonparasitized males, there was no relationship between plumage brightness and body condition, plumage brightness was negatively associated with body condition for parasitized males (Fig. 3A). As stated above, such a pattern might indicate that the detrimental effect of blood parasites forces males to trade the allocation of carotenoids between immune defence against the parasites and plumage elaboration.

Another result for plumage brightness that is similar to our findings for plumage coloration is the lack of a significant main effect of testosterone and the significant interaction between testosterone and HLR (Table 1), with males that did not suffer from high stress levels showing a positive relationship between testosterone levels and plumage brightness, whereas males suffering from high stress levels showed a negative relationship between testosterone levels and plumage brightness (Fig. 4A). As discussed above for plumage coloration, this effect might be a consequence of an immunosuppressive effect of testosterone, where males with both high hormone levels and high stress levels have to allocate carotenoids away from plumage elaboration to support the suppressed immune system.

The most obvious difference regarding the factors affecting plumage brightness and plumage coloration

is that body condition appears to have a stronger effect on plumage brightness than on coloration. This is reflected by the significant main effect of body condition on plumage brightness together with two significant interaction effects involving body condition that were not found for plumage coloration (Table 1). This indicates that brightness can be used by females as a visual cue for individual condition when assessing potential mates. Whereas most correlational and experimental studies have revealed significant relationships between feather colour (i.e. chroma and/or hue) and body condition rather than between brightness and body condition (Hill, 2002), there are some studies demonstrating that brightness is a better indicator of male quality than plumage coloration. In a study on golden-collared manakins, *Manacus vitellinus*, Stein & Uy (2006) found that collar brightness was correlated with body size and mating success, whereas PCs reflecting chroma and hue did not show such correlations. Likewise, in red fodies (*F. madagascariensis*) only brightness, but not chroma of male breast plumage, was significantly related to body condition (Estep *et al.*, 2006), and a study on greenfinches (*C. chloris*) showed that males with brighter yellow feathers were able to mount a stronger immune response to a novel antigen than duller conspecifics (Saks *et al.*, 2003). Shawkey & Hill (2005) demonstrated that structural components of feather barbs responsible for the underlying white structural colour of pigmented feathers can strongly influence brightness of carotenoid-containing tissues. While the production mechanisms and potential metabolic costs of such white structural coloration remain virtually unknown, it is possible that plumage brightness of carotenoid-based plumage ornaments is an important but over-looked indicator of quality in birds. Clearly, more studies are required to investigate the signalling value of plumage brightness (in contrast to plumage coloration) and the underlying mechanism responsible for inter-individual variation in brightness of such carotenoid-based plumage ornaments.

The significant interaction between HLR and body condition confirms the patterns described before. For males in very bad body condition (i.e. body condition in the lowest quartile of the distribution), there is a positive relationship between plumage brightness (PC1 scores) and HLR (Fig. 5), again indicating a trade-off with individuals being able to allocate resources either in plumage brightness or in health maintenance, but not both. By contrast, for males in average or good body condition (i.e. body condition in the upper three quartiles of the distribution), the relationship between HLR and plumage brightness is slightly negative (Fig. 5), suggesting that males in good body condition have more available resources that can be invested in the immune system, allowing them to simultaneously produce bright feathers at the same time as keeping their stress level and immune system on a suitably functioning level. This again illustrates the difficulty of assessing male quality based on plumage characteristics alone because males in very different health conditions can express the same level of plumage ornamentation.

Finally, there was a significant effect of the interaction between day of capture and body condition on brightness. When in good condition, male plumage brightness did not change over the course of the breeding season, whereas, for males in bad body condition, brightness declined over the season. One possible explanation for this pattern would be that plumage brightness is affected by preening behaviour and that males in good body condition are likely to spend more time preening; these males might thus better be able to maintain their plumage brightness throughout the breeding season compared to males in worse body condition.

Conclusions

The present study has revealed a complex pattern of factors determining carotenoid-based plumage ornamentation in red bishop males. In general, plumage brightness and plumage coloration appear to be affected by the same variables and factors, with plumage brightness probably being a better indicator of male quality than plumage coloration. However, the relationships between plumage traits and factors such as HLR and blood parasite load varied considerably among seasons, indicating a strong effect of environmental conditions. Furthermore, several significant interactions suggest that the overall condition of males (body condition, stress levels, blood parasite load) can strongly affect the association pattern between plumage traits and other factors, with high stress levels or parasite loads, as well as bad body condition, forcing males to allocate resources away from plumage elaboration to the maintenance or enhancement of immune functions. Thus, males that are in generally good condition can afford to invest in plumage ornamentation without obvious detrimental effects on health, whereas males in bad overall condition face a trade-off: they can invest either in plumage ornamentation or in the maintenance of body condition, health and immune function, but not simultaneously in both.

There are two conclusions that can be drawn from these results. First, females cannot use plumage traits alone to gather information on male quality, but have to assess additional factors that advertise general male health status (possibly through obser-

vations of male behaviour). Otherwise, it would be difficult for females to distinguish between males in bad condition that invest most of the available energy in plumage ornamentation at the cost of body maintenance, and males in general good health that can afford to invest in plumage ornamentation without detrimental effects on health, given that both groups of males could display the same degree of plumage ornamentation. Second, studies aiming to investigate the signalling value of plumage traits should include several indices of health and should cover more than one season to be able to evaluate the complex and variable pattern of associations between plumage ornamentation and other male traits.

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