ECOPHYSIOLOGY

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Carotenoid-based plumage coloration of male greenfinches reflects health and immunocompetence

Abstract Hypotheses of parasite-mediated sexual selection (PMSS) propose that elaborate male ornaments have evolved due to female preferences. Females would benefit from mating with more ornamented males if males' ornamentation signals their health status and ability to provide parasite resistance genes for the offspring. Carotenoid-based plumage coloration of birds has been hypothesised to honestly reflect an individual's health status due to trade-off in allocation of carotenoids between maintenance and signalling functions. The prediction of this hypothesis, namely that individuals with brighter plumage are able to mount stronger immune responses against novel antigens and reveal generally better health state, was tested in captive male greenfinches (*Carduelis chloris*). Greenfinches with brighter yellow breast feathers showed stronger humoral immune response against novel antigen (SRBC) while no relationship between plumage coloration and an estimate of cell-mediated immune responsiveness (PHA response) was detected. Elaborately ornamented individuals had better general health state as indicated by the negative correlations between plumage brightness and heterophil haemoconcentration. Consistent with the concept of PMSS, these results suggest that carotenoid-based plumage coloration in greenfinches honestly signals immunocompetence and health status.

Keywords *Carduelis chloris* · Carotenoids · Immune response · Leukocytes · Plumage colour

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Introduction

To explain the evolution of extravagant male ornaments, Darwin (1871) proposed the theory of sexual selection, which he separated from the general theory of natural selection. According to this, elaborate male ornaments could evolve if females would prefer ornamented males as mates. To explain the mechanisms responsible for such female preferences, a number of hypotheses have been proposed. According to the "good genes" hypothesis, the females' preference for elaborate male ornaments could evolve if these ornaments serve as indicators revealing the quality of the male (review in Andersson 1994). The honesty of the indicator traits would be ensured by the high cost (developmental, maintenance, behavioural, etc.) of such signals, so the individuals of inferior quality could not afford to cheat. The idea of parasite-mediated sexual selection (PMSS) was established by Hamilton and Zuk (1982), who hypothesised that the individual's ornamentation is revealing its ability to resist currently prevailing parasites. Under this scenario, males which are more resistant to parasites and in better health are able to invest more resources into sexual display. Such healthy males would be capable of higher paternal investment and/or transmit less parasites during physical contact. Furthermore, if resistance is heritable, then females would also benefit by obtaining resistance genes for their offspring (review in Andersson 1994).

Carotenoids have been suggested as playing a major role in PMSS, especially among birds where carotenoid-based ornaments are disproportionately common (reviews in Gray 1996; Møller et al. 2000). Because birds cannot synthesise carotenoids and have to acquire them through the food chain (e.g. Fox 1979) carotenoids may appear dietary limiting factors of expression of such signals (e.g. Hill 1992; Hill et al. 1994). For instance, dietary addition of carotenoids has been shown to enhance the brightness of coloration of the ornaments (e.g. Hill 1992; Grether et al. 1999; Saino et al. 2000). Furthermore, carotenoids (along with other antioxidants) are required in various roles for several physiological mechanisms, like endo-

crine and nervous systems, and play important roles in immunoregulation and immunostimulation, lymphocyte proliferation, free radical scavenging and detoxification (review in Møller et al. 2000). Hence, one might expect a trade-off between investment of carotenoids in maintenance and in ornamentation because individuals that are forced to fight infections during formation of carotenoid-based ornaments have less carotenoids available for developing signal traits. (e.g. Lozano 1994; Olson and Owens 1998; Møller et al. 2000).

Despite the considerable evidence supporting the idea that carotenoid-based signals honestly reflect an individual's health status and ability to resist parasites (review in Møller et al. 2000), some studies have not found the predicted negative relationship between the expression of carotenoid-based signals and parasite loads (e.g. Weatherhead et al. 1993) or have demonstrated that more ornamented birds are actually more infected (e.g. Burley et al. 1991; Seutin 1994; Dufva and Allander 1995; Korpimäki et al. 1995). Inconsistent relationships between carotenoid-based ornaments and host infection status can arise because an individual's infection status does not necessarily always reflect its ability to resist parasites. This is because not only resistant individuals, but also susceptible individuals which have not been exposed to the parasites, appear parasite-free (Clayton 1991; Merilä et al. 1999). Furthermore, most of the studies in this field have focused on a few taxa of parasites with weak or no effect on the fitness of their host (Hamilton and Poulin 1997; Møller et al. 2000). Therefore, it has been argued that individual immunocompetence, i.e. an ability to rise an immune response to a novel antigenic challenge, appears a more relevant measure of parasite resistance than actual parasite counts (Apanius 1998; Møller et al. 2000). Under this scenario, individuals with the most elaborate sexual ornaments would also have more efficient immune systems. This prediction has been supported by meta-analysis by Møller et al. (1999) showing that the expression of secondary sexual characters was more strongly related to measures of immune function than to measures of parasite load.

To date, few studies have assessed the relationships between carotenoid-based ornamentation and an individual's ability to mount immune response against novel antigens. All these studies have assessed carotenoid-based ornamentation of the living tissues such as combs (Zuk et al. 1995; Zuk and Johnsen 1998; Verhulst et al. 1999), gapes (Saino et al. 2000) or peaks (Preault 1999, cited in Møller et al. 2000). Regrettably, there appear to be no studies of relationships between individual immunocompetence and carotenoid-based plumage coloration, despite the feather ornaments being the most widespread form of display amongst birds (Andersson 1994). The feather ornaments are, however, particularly interesting in the context of PMSS because once the carotenoids are deposited in such a metabolically inactive tissue, they become unavailable for enhancement of immune function and/or detoxification (Lozano 1994; Olson and Owens 1998). Hence, one might expect the carotenoid-based feather ornaments to be a particularly honest signal of individual disease resistance because individuals currently fighting infections are expected to have less carotenoids available for deposition into plumage. Moreover, since in most species moult occurs long before using feathers in sexual display, carotenoid-based plumage coloration can be predicted to signal especially long-term aspects of an individual's quality. Choosing a mate on the basis of plumage coloration makes sense only when an individual's plumage colour (which reflects its past health state) also manifests its current and general disease resistance.

The aim of this study is to test whether the plumage brightness of captive male greenfinches (*Carduelis chloris*) reflects their ability to produce an immune response to novel antigens and their general health state. Greenfinches are medium-sized (ca. 28 g) gregarious seed-eating passerines native to the western Palearctic region (Cramp and Perrins 1994). Males are larger and more colourful (Svensson 1992; Merilä et al. 1999), old males being olive-green on their back, with a bright yellow breast (greenish-yellow in some geographic regions), less so on the belly and rump, and yellow markings on primaries, primary coverts and the sides of the tail feathers (Cramp and Perrins 1994). Females are smaller, more olive-brown and yellowish buff, having faint brown streaks on the back and lacking full yellow tints in their plumage. Moult duration is 13–15 weeks from late July to early November (Cramp and Perrins 1994). The mating system is mainly monogamous, but a significant degree of polygamy occurs, at least in some populations (ca. 24% of males in a population in southern England; Eley 1991). During the mating season, males perform conspicuous song-flights, butterfly-flights and other kinds of display, exposing their yellow breast area to females (Cramp and Perrins 1994). Several clutches are laid each year and, hence, the period of sexual activity is longer than in most other northern temperate-zone passerine birds. Further, the testis size is larger than expected from body weight, suggesting that sperm competition may be relatively intense in this species (Merilä and Sheldon 1999; Møller 1991). The male plumage brightness (measured by visual scoring) has been shown to be a sexually selected trait as more brightly coloured male greenfinches tend to be favoured by females as mates (Eley 1991). It has also been shown that males with more yellow ornamental feathers are less likely to be heavily infected with haemoparasites (Merilä et al. 1999) and have higher virus clearance rates (Lindström and Lundström 2000). The yellow pigments in the plumage of greenfinch consist of carotenoids: all trans 3-hydroxy-ε,ε-carotene-3'-one, all trans ε,ε-carotene-3,3'-dione, and lutein (probably the precursor of all the carotenoids found in the plumage; Stradi et al. 1995).

To estimate humoral immunocompetence, we used sheep red blood cell (SRBC) injection assay. Immune response against this multigenic antigen involves both T and B lymphocytes (e.g. Munns and Lamont 1991) and the antibody production can be easily assessed by a

haemagglutination assay. Cell-mediated immune responsiveness was estimated on the basis of quantification of swelling response to intradermally injected plant-lectin phytohaemagglutinin (PHA; Goto et al. 1978; Alonso-Alvares and Tella 2001). An individual's general health status was assessed on the basis of leukocyte profiles and concentrations, known to reflect infection and/or inflammatory process (e.g. Dein 1986) and showing high long-term individual consistency in captive greenfinches (Hõrak et al. 2002). We predicted that individuals with brighter carotenoid-based feathers would have stronger immune responses against novel antigens and lower leukocyte (particularly heterophil) haemoconcentrations (as an indicator of low levels of infection, inflammation, or stress).

Materials and methods

A total of 32 male greenfinches was caught in mist-nets in the Kabli ornithological station in SW Estonia (58°01'N, 24°27'E) between 24 September and 17 October 2001. The birds were transported to Tartu and housed in individual indoor cages (27×51×55 cm). The work was carried out in two series: from 10 October to 4 December (hereafter "autumn") and from 2 to 17 March (hereafter "spring"). The average temperature in the aviary was 13.9±1.6 SD °C during the autumn and 13.6±1.2 SD °C during the spring. The birds were fed ad libitum with peeled sunflower seeds and additionally about three to four rowan berries were provided to each bird during the autumn experiment. Fresh tap water was always available. During both study periods birds were held in constant light conditions: 11 h of artificial light followed by 13 h of darkness which was the natural day-length at the time. Between the experiments, birds were held in natural day-length cycle. The study was conducted under a licence from the Estonian Ministry of the Environment and all the birds were released to their natural environment after the experiment.

Research protocol

During autumn, 32 birds were sampled for haematological measurements. Birds were blood sampled at 4-day intervals (on the first, fourth and eighth day of the experiment) around 0800 hours, and the averages of three measurements of each variable were used in statistical analyses. On day two, 16 individuals were injected with a suspension of SRBCs diluted in sterile isotonic saline to induce the humoral immune response, while the rest of birds received an injection of the same amount of isotonic saline. Since immune challenge did not affect any of the leukocytic variables measured (Peeter Hõrak et al., unpublished data), data from SRBC and saline-injected individuals were pooled for analyses of the relationship between leukocytic parameters and plumage coloration. Blood samples (ca. 150 μl) for assessment of immune response were obtained 8 days after the injection. Cell-mediated immune responsiveness was estimated on the basis of PHA injection assay (see analyses) in the morning following the last sampling. Immune challenged individuals were released after autumn measurements, leaving us 16 birds (two of which died before spring for unknown reasons) for spring. In spring, seven birds received similar antigen injections as in autumn. Since there was no significant difference in SRBC antibody titre between the autumn and spring (3.56±2.19 SD autumn vs 2.00±2.33 SD spring) ($Z_{16,8}$=1.4, P=0.159), the data were pooled over both study periods for analyses of the relationships between plumage coloration and SRBC antibody titres. Before releasing the birds, about two to four feathers were collected from their breast area to assess plumage brightness.

Analyses

High leukocyte counts have been interpreted to reflect an individual's current investment in immune defence (e.g. Møller 1998; Nunn et al. 2000). In particular, elevated leukocyte count (leucocytosis) is characteristic for inflammatory processes in response to microbial and macroparasite infections (e.g. Dein 1986). Specifically, we concentrated on the two most numerous leukocyte types, namely heterophils and lymphocytes. Heterophils are non-specific phagocytosing cells that enter the tissues during the inflammatory response. Lymphocytes elicit pathogen-specific immune response. T-lymphocytes (which comprise the majority of circulating lymphocytes) play a key role in cell-mediated immunity, while B-lymphocytes that produce immunoglobulins are primarily responsible for antibody-mediated or humoral immunity. An index comprising the relative abundance of both lymphocytes and heterophils is the heterophil/lymphocyte (H/L) ratio, which is widely used to estimate stress in poultry (e.g. Gross and Siegel 1983; Maxwell 1993) and also in wild birds (Trust et al. 1994; Birkhead et al. 1998; Schulz et al. 1998; Camplani et al. 1999; Totzke et al. 1999). For counting leukocytes, a drop of blood was smeared on individually-marked microscope slides, air-dried, fixed in absolute methanol, and stained with azure-eosin. The proportion of different types of leukocytes was assessed on the basis of examination of 100 leukocytes under 1,000× magnification under oil immersion. Estimates of the total white blood cell count (WBC) were obtained by counting the number of leukocytes per approximately 10,000 erythrocytes. Differential leukocyte counts were obtained by multiplying their proportions with WBC. The repeatabilities of leukocyte counts obtained in this method were found to be reasonably high and significant (Ots et al. 1998). Blood smears were also scanned for the presence of blood parasites and the birds were checked for the presence of the Sindbis virus antibodies (see Lindström and Lundström 2000 for methodology); all individuals appeared negative.

To induce humoral immune response, birds were injected with a 50-μl suspension of 40% SRBCs in isotonic saline into their pectoralis muscle. Prior to injection, SRBCs were double washed and resuspended in saline in order to achieve the desired concentration. Serum was separated by centrifugation at 3,000 rpm for 10 min and preserved at −20°C until analysis. Immune response (anti-SRBC antibody titre) was measured using a haemagglutination assay (Wegmann and Smithies 1966; Lawler and Redig 1984) in 96-well microplates. A 12.5-μl aliquot of serum was added to 87.5 μl of saline in the first well of a plate and serially diluted using 50 μl of saline (0.5, 0.25, etc.). Then 50 μl of 1% suspension of SRBCs in saline was added to all wells. The microplates were incubated at 37°C for 1 h. Titre was scored as the inverse of the dilution that contained sufficient antibodies to haemagglutinate SRBCs (hence, the higher the titre, the stronger the immune response).

Wing web swelling in response to intradermal injection of a plant lectin PHA is routinely used as an index of the efficiency of cell-mediated immunity in different bird species (see Smits et al. 1999; Martin et al. 2001). PHA injection assay (Goto et al. 1978) was used to evaluate cell-mediated immunity in vivo following the simplified protocol of Smits et al. (1999); [see also references in Alonso-Alvarez and Tella (2001) for a review of studies using this technique]. In the morning following the last sampling, birds were injected intradermally in the wing web (the patagium) with 0.2 mg of PHA (Sigma, L-8754, St. Louis, Mo.) in 0.04 ml of isotonic saline. The thickness of wing webs was measured immediately before and 24 h after injection in inoculated sites using a spessimeter (SM112, Tclock, Japan) with an accuracy of 0.01 mm. Swelling of the wing web (PHA-response) was calculated as the difference in thickness of the wing web prior to and 24 h after injection. Following Smits et al. (1999) we did not use a control injection. Both before and after PHA injection, the thickness of the wing web was measured three times, and the average of these three measurements was used in calculations. The measurement error for the PHA response was reasonably low as the repeatability (Lessells

and Boag 1987) of the wing web index, based on three consecutive measurements, was 0.92 ($P<0.0001$, $n=40$).

For assessment of the carotenoid-based plumage conspicuousness we used the "brightness of yellow", i.e. the measure of total amount of light reflected from the feathers in the yellow part of spectrum [the sum of reflectances at all measured wavelengths between 550 and 625 nm; see Endler (1990) and Grill and Rush (2000) for details]. This measure was highly correlated with total brightness of the feathers, measured over the visual part of the spectrum from 400 to 700 nm ($r=0.97$, $P<0.0001$, $n=30$).

Analysis of plumage colour was performed on two to four feathers, collected from the standard position on the breast: the midpoint between the middle part of the sternum and the edge of the wing. Feathers were placed into a plastic bag and stored in the dark until measurements were carried out. Colour was measured in an area of the visible surface of the feather of approximately 1 mm^2 using a spectrometer (Ocean Optics S2000). Two feathers of the same individual were placed on top of each other on the black background, and the mean of three scores for each set of two feathers was averaged to obtain the individual values. Light from a halogen light source (Ocean Optics LS-1) was transferred to the feather through a quartz fibre optic (Ocean Optics), reaching the feather at 90°. The sampling optic was placed at 45° to the surface of the sample and connected to a spectrometer by quartz fibre optic cable. Data from the spectrometer were digitised by a DAQ Card 700 and passed into a computer with appropriate software (OOIBase). The measurements were relative and referred to a standard white reference tile (WS-2) and to the dark. Each pair of feathers provided a measure of transmittance for each 1-nm interval in the range of 400–700 nm. Repeatability of measurements of brightness of yellow for one set of feathers was 0.70 ($P<0.001$, $n=30$), for two sets of feathers of the same bird the repeatability was 0.72 ($P<0.001$, $n=16$).

To examine the relationships between plumage coloration, leukocyte counts and immune responsiveness, Pearson correlation coefficients were calculated. All given P-values (except when comparing the SRBC antibody titre between the autumn and spring) are calculated for directed tests (Rice and Gaines 1994) using the convention that $\gamma/\alpha=0.8$, since we expected brighter birds to be in superior condition. All variables were tested for normality (Lillefors test). Since heterophil and lymphocyte counts and ratios were not normally distributed, they were log$_n$-transformed to obtain normality.

Results

Greenfinches with brighter yellow breast feathers showed stronger humoral immune response ($r=0.45$, $P=0.022$, $n=22$; Fig. 1). The brightness of feathers was correlated negatively with heterophil counts ($r=-0.41$, $P=0.016$, $n=30$; Fig. 2). Brightness did not however correlate significantly with lymphocyte count ($r=-0.14$, $P=0.295$, $n=30$), H/L ratio ($r=-0.27$, $P=0.096$, $n=30$) or WBC ($r=-0.27$, $P=0.095$, $n=30$). Albeit the cellular immune response to PHA was significantly and positively correlated to the strength of humoral immune response ($r=0.45$, $P=0.028$, $n=20$; Fig. 3), the correlation between the plumage brightness and the PHA response was not significant ($r=-0.13$, $P=0.346$, $n=25$).

Discussion

Male greenfinches with brighter yellow breast feathers mounted significantly stronger humoral immune response to SRBCs than duller individuals (Fig. 1). To our
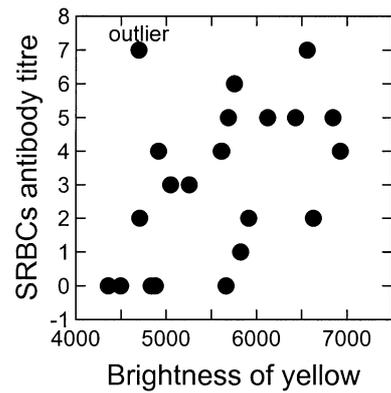

Fig. 1 Relationship between the brightness of yellow feathers and the SRBCs antibody titre feathers in the greenfinch (*Carduelis chloris*). If the marked outlier (standardised residual=2.39) is excluded from the analysis then $r=0.61$, $P=0.002$, $n=21$
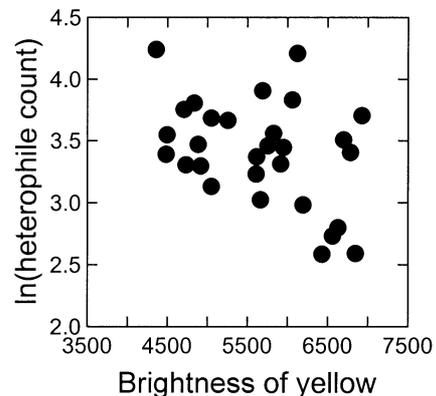

Fig. 2 Relationship between the brightness of yellow feathers and heterophil count (per ca. 10,000 erythrocytes)
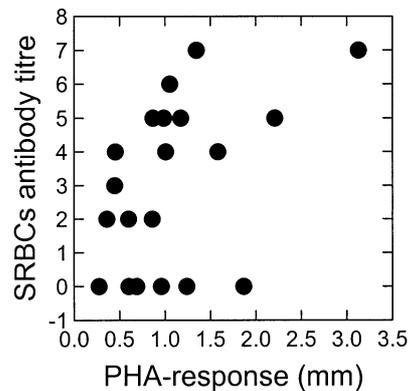

Fig. 3 Relationship between the immune response to phytohaemagglutinin (PHA response) and the SRBC antibody titre

knowledge, this is the first evidence that carotenoid-based coloration of metabolically inactive ornamental tissues reflects humoral immunocompetence. The prediction, that more ornamented males have less parasites and

more efficient immune system, has been tested by a number of studies (reviewed in Møller et al. 2000). However, the relationships between immunocompetence and carotenoid-based ornaments have been studied only on metabolically active ornamental organs, like combs (e.g. Zuk et al. 1995), ceres, lores, legs (Bortolotti et al. 1996), or gapes (Saino et al. 2000). As shown by Saino et al. (2000) in nestlings of barn swallow (*Hirundo rustica*), such ornaments may reflect short-term and reversible changes in individual condition. When the immune system of nestlings was challenged by injection with SRBCs, the carotenoid-based gape coloration was reduced, which could be restored by supplemental feeding with lutein. In contrast to the metabolically active tissues, carotenoids allocated into feathers are no longer available for the immune system. Thus, investing carotenoids into feathers is likely to involve greater costs as it may lead to resource shortages for other vital physiological functions (e.g. Lozano 1994; Olson and Owens 1998). Furthermore, the feathers are usually used for display a long time (often several months) after their formation. So, if the carotenoid-based plumage coloration is used in mate choice, then it has to reflect some persistent component of individual phenotypic quality. Our results suggest that individuals who were able to mount stronger antibody response against SRBCs had been able to invest more carotenoids into development of plumage signals during autumnal moult than these mounting weaker immune response. This result compares favourably with that of Lindström and Lundström (2000), who found that male greenfinches with larger areas of yellow ornamental feathers had higher Sindbis virus infection clearance rate. Additionally, the association between greenfinch plumage coloration and blood parasite (*Haemaproteus chloris*) infection intensity was found by Merilä et al. (1999). All these findings are consistent with the concept of PMSS, predicting that more immunocompetent (less parasitised) males are more ornamented (Zahavi 1975; Hamilton and Zuk 1982; Grafen 1990; Andersson 1994). It should be noted, however, that the positive correlation between SRBC response and plumage coloration cannot be explicitly interpreted to support the original Hamilton-Zuk hypothesis of PMSS because the crux of this hypothesis is the concept of signalling of heritable parasite resistance due to an evolutionary arms race between hosts and parasites. The hypothesis predicts that the most ornamented males are those who can signal their resistance to currently prevailing virulent parasite strains. This study, however, demonstrated a correlation between ornamentation and a measure of general humoral immunocompetence against novel antigen. At present we do not know whether between-individual differences in SRBC responses in greenfinches have a genetic basis. However, studies in barn owls (*Tyto alba;* Roulin et al. 2000) and domestic fowl (e.g. Siegel and Gross 1980; Dunnington and Siegel 1984; Parmentier et al. 1996, Boa-Amponsem et al. 1998, 2001) have demonstrated a substantial additive genetic variation for antibody response to SRBCs. Selection for increased SRBC-response in poul-

try has resulted in an increased response to some pathogens (Newcastle disease virus, *Escherichia coli*, *Staphylococcus aureus*; Gross et al. 1980). Such correlated responses could be due to altered functions of the immune system, linkage with disease resistance genes, or cross reactivity of the antigenic determinants of SRBC and the infectious organisms (e.g. Boa-Amponsem et al. 1998). However, selection for high SRBC antibody responsiveness can also result in reduced resistance to some pathogens, such as marble spleen disease virus, and lower growth rates and postponed sexual maturity (Boa-Amponsem et al. 1998). Such negative genetic correlations may appear an additional reason for maintenance of genetic variation in immune responsiveness and parasite resistance in natural populations.

Although the plumage brightness of greenfinches correlated positively with a measure of humoral immunocompetence, we were not able to demonstrate a similar relationship between carotenoid-based plumage colour and an estimate of cell-mediated immune function. The latter result, however, does not contradict the general concept of PMSS given that different ornaments might reveal different individual qualities (Møller and Pomiankowski 1993). For instance, a recent study by Møller and Petrie (2002) showed that train length in peacocks reflected cell-mediated and humoral immunity, whereas the size of ocelli reflected cell-mediated immunity.

The result that SRBC antibody response in greenfinches was significantly correlated with the magnitude of cellular immune response to PHA suggests that both types of immune response possess a certain amount of variation, which can be ascribed to current physiological condition of the individual. This is not surprising because the magnitude of PHA response has been shown to be particularly sensitive to short-term variation in individual nutritional state (review in Alonzo-Alvarez and Tella 2001; Lifjeld et al. 2002) and also the magnitude of humoral immune response to foreign erythrocytes has been shown to depend on external conditions (e.g. Moore and Siopes 2000).

In addition to the positive correlations between humoral immunocompetence and plumage brightness, our study also revealed negative correlation between plumage brightness and concentration of circulating heterophils. Heterophils (functional analogues of mammalian neutrophils) are highly phagocytic leukocytes capable of a broad spectrum of antimicrobial activity (e.g. Dein 1986; Harmon 1998). Heterophils form the first line of cellular defence of an organism during the acute inflammatory response and their concentration in the bloodstream is known to rise in response to microbial pathogens and corticosterone administration (review in Harmon 1998). Our results thus suggest a poorer general health status of male greenfinches with duller breast plumage. Since heterophil haemoconcentration showed high repeatability between autumn and spring values in our captive greenfinches (repeatability =0.62; Hõrak et al. 2002), we can conclude that this variable reflects relatively long-term components of an individual's gen-

eral health state. Hence, it is possible that correlations between heterophil concentration and plumage colour arise because individuals suffering microbial infections during moult had to use more carotenoids for immunostimulation and/or repair functions and therefore had less carotenoids available for deposition into their plumage.

In conclusion, our results suggest that the carotenoid-based ornamental plumage of male greenfinches might indeed have evolved as an indicator of an individual's general health state and ability to resist parasites as predicted by the concept of parasite-mediated sexual selection. However, the questions about mechanisms relating immunocompetence and ornament expression and whether and how such patterns are inherited remain to be answered in future studies.

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