Animal Behaviour xxx (2011) 1–7

Contents lists available at SciVerse ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

Phytohaemagglutinin skin-swelling test in scarlet rosefinch males: low-quality birds respond more strongly

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ARTICLE INFO

Article history:
Received 24 May 2011
Initial acceptance 14 July 2011
Final acceptance 21 September 2011
Available online xxx
MS. number: 11-00439R

Keywords:
Carpodacus erythrinus
condition
ecoimmunology
ecological immunology
health signalling
immunocompetence
ornamentation
phytohaemagglutinin
ptilochronology
scarlet rosefinch

The phytohaemagglutinin (PHA) skin-swelling test is one of the most widely used methods for cell-mediated immunity measurement in immunoecology. Although several studies have investigated the condition-dependent traits associated with the magnitude of cutaneous inflammatory response to PHA, the results concerning signalling of the responsiveness through ornamental traits are still controversial. This is especially true for carotenoid-based feather ornamentation in birds. We therefore examined the linkage between several condition-dependent traits, including the red ornamental coloration of the plumage, and the magnitude of the PHA-induced immune response in scarlet rosefinch, *Carpodacus erythrinus*, males. Our results show two important aspects of the PHA-induced inflammation in this species. First, histological analysis showed that the swelling response was dependent on basophil activity. Second, the magnitude of the response (increase in patagium thickness) was associated with individual size, carotenoid-based ornamental coloration and a ptilochronological marker of feather growth at the time of moulting (mean growth bar width), thus mirroring the long-term quality of the individual. The positive linkage between the individual size or mean growth bar width and the PHA response suggests an association between the magnitude of the response and individual metabolic rate. However, as the magnitude of the response was also related negatively to ornament saturation and positively to ornament lightness, our results indicate stronger responsiveness in inferior males. Highly ornamented, healthier individuals recruited fewer basophils into the inflamed tissue causing less intense swelling. To our knowledge, this study is the first to show a negative association between carotenoid-based plumage coloration and the magnitude of the PHA-induced immune response.

Many ornamental traits important in mate choice are thought to signal individual health and vigour (Andersson 1994; Vinkler & Albrecht 2010). This aspect of sexual selection currently attracts much attention from behavioural ecologists, thus contributing to the fast development of immunoecology as a research discipline. Among the immunological methods utilized in behavioural studies only a few have become popular over the past two decades. The phytohaemagglutinin (PHA) skin-swelling test is undoubtedly one of these techniques and is now widely used in birds and mammals (Kennedy & Nager 2006; de Bellocq et al. 2007; Ardia & Schat 2008). A lot of potentially valuable data have been gained on the basis of this particular method. The test has been adopted in research devoted to the exploration of the relationships between immunity and metabolism (e.g. Martin et al. 2003), oxidative damage (e.g. Hõrak et al. 2007), carotenoid-based immunomodulation (e.g. Hõrak et al. 2006), antioxidant capacity (e.g. Hõrak et al. 2007; Perez-Rodriguez et al. 2008), immunosenescence (e.g. Haussmann et al. 2005) and mate choice (e.g. Johnsen et al. 2000; Perez-Rodriguez et al. 2008).

The PHA skin-swelling test is based on artificial activation of the immune defence with plant lectin PHA which is (during the first application) novel to the host and possesses specific immunostimulatory properties (Vinkler et al. 2010a). Currently, the most common test protocol is the one proposed by Smits et al. (1999) in which the response index is calculated as the thickness of the swollen tissue at a defined time after the PHA treatment minus the tissue thickness before the PHA injection. Although the results of the PHA skin-swelling test are commonly referred to as a measure of acquired immunity, often named as the 'T-cell-mediated immunocompetence' (most recently, e.g. Tella et al. 2008; Hawley et al. 2009; Kilgas et al. 2010), recent results suggest that this response might in fact be a nonspecific inflammation without T-cell-dominant involvement (Martin et al. 2006; Sarv & Hõrak 2009; Vinkler et al. 2010a). The intensity of the response has been shown

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doi:10.1016/j.anbehav.2011.10.001

to be heritable in some species (Bonneaud et al. 2009), but not in others (Christe et al. 2000). This is in concordance with current knowledge suggesting that both environmental and genetic factors significantly influence the outcome of an immune response (Buchholz et al. 2004; Vinkler & Albrecht 2009; Beldomenico & Begon 2010). Genetically codetermined traits should be at least partially stable in time. However, in the case of PHA treatment it is difficult to assess individual consistency because any repeated application of PHA causes an adaptive immune response to PHA (Tella et al. 2008) distinct from the immunological processes involved in the response after the first PHA injection. In other words, repeating the same procedure in the same group of individuals will not necessarily provide a comparable result.

Nevertheless, the stability of this immunological trait might be estimated by assessing the stability of traits correlated with the response intensity. Evidence suggests that the magnitude of the skin swelling induced by PHA may be reflected by colourful ornamental traits such as carotenoid-based bill (Faivre et al. 2003b; McGraw & Ardia 2003) or skin coloration (Mougeot 2008; Perez-Rodriguez et al. 2008; Bonato et al. 2009). Although these traits seem to show some individual consistency (Perez-Rodriguez 2008), it is also known that coloration may undergo rapid changes as a result of changes in an individual's health (Faivre et al. 2003a; McGraw & Ardia 2003; but see also Biard et al. 2009 for contradictory evidence). Therefore these 'dynamic' ornamental traits are often viewed as more reliable indicators of actual condition and health than feather ornaments that are produced only during the annual moult (Faivre et al. 2003a). Many colourful avian ornaments are, however, feather based and much less is currently known about their relationship to the PHA-triggered immune response. Some studies have linked PHA-induced skin swelling to melanin-based plumage ornamentation (Gonzalez et al. 1999a; Jacquin et al. 2011) but others have found no such pattern (Gonzalez et al. 1999b). A similar pattern has also been described for the structurally based feather UV coloration (Parn et al. 2005; Griggio et al. 2010). Nevertheless, melanin-based and, in some species, also structurally based ornaments reflect the social status of their bearer more than its condition and health (McGraw et al. 2003; Badyaev & Young 2004). On the other hand, carotenoid-based ornaments are commonly assumed to be valuable indicators of the health and parasite resistance of individuals (see e.g. Vinkler & Albrecht 2010). Unfortunately, there is little evidence on this subject and no clear pattern for the association between carotenoid-based feather ornamentation and the magnitude of the PHA-induced immune response (Navara & Hill 2003; Saks et al. 2003b). The same is also true for associations between other long-term condition-related traits such as ptilochronological traits and the outcome of the PHA skin-swelling test.

In the present study we focused on the possible linkage between the magnitude of the PHA-triggered immune response 6 h after PHA application and several traits associated with long-term condition in the scarlet rosefinch, *Carpodacus erythrinus*. Since the red breast plumage in this species seems to be an important predictor of individual fitness (Albrecht et al. 2009), we decided to focus on this trait. In rosefinch males captured before breeding we analysed three components of this ornamental coloration: hue, saturation and lightness. Furthermore, we assessed the mean growth bar width in a rectrix as a ptilochronological marker of the speed of feather growth (Grubb 2006) and recorded measures of morphometric indicators of condition such as size and size-standardized weight (body mass). To clarify the mechanism underlying the PHA-induced immune response in this species, we also performed histological analysis of the swollen tissue in a subset of males to characterize the cellular composition of the inflammation.

METHODS

Field Procedures

In 2006–2009 we mist-netted newly arrived scarlet rosefinch males (in the second half of May, before breeding) in the Vltava river valley, Šumava National Park, Czech Republic (48°49′N, 13°56′E; for more detailed description see Albrecht 2004). In total, 45 males 3 or more years old (considered as adult based on their plumage coloration) were caught and examined according to the following protocol. First, we weighed the male using a Pesola spring balance (accuracy 0.5 g) and collected the second outermost rectrix from the left side of the tail for the ptilochronological assay. We then carried out the PHA treatment. In this study we adopted the simplified protocol described by Smits et al. (1999) with the following modifications. The treatment dosage we used was 0.10 mg of PHA-P dissolved in 20 μl of DPBS (product numbers L8754 and D5652, respectively; Sigma–Aldrich, St Louis, MO, U.S.A.; see Vinkler et al. 2010a). The thickness of the tissue of the left wing-web (patagium) was measured and the PHA solution was injected into the middle of the patagium. After the injection each bird was placed individually into a cage (41 × 23 cm and 23 cm high) with access to natural food (willow and dandelion seeds) and water ad libitum, in a calm shady place with no visual contact with the surroundings or with other birds. Cages were periodically checked to ensure that all birds were feeding normally and did not show any signs of stress. The magnitude of the swelling reaction was measured after 6 ± 0.5 h to avoid any negative impact of caging on the male's natural mating performance (for the usage of a 6 h period see e.g. Møller et al. 2003; recently Bonato et al. 2009 have shown that there is no statistical difference in the PHA response between 6 h and 24 h after injection). From the histological point of view, we investigated the early phase of the PHA-induced inflammation. We measured the tissue thickness three times with (accuracy 0.01 mm) and took the average for further analysis (repeatability of the measurement was $r = 0.96$). The PHA-induced swelling response index was later calculated as the average tissue thickness 6 h after the treatment minus the average thickness before the PHA injection. In 2009, we collected a biopsy sample of the tissue from the middle of the inoculation site immediately after measuring the thickness of the swelling. We used a tissue corer 2.0 mm in diameter (FST 18035-02, Fine Science Tools GmbH, Heidelberg, Germany) and fixed the sample in 10% formalin for later histological analysis (Vinkler et al. 2010a). We then weighed the male again and measured the tarsus length (accuracy 0.01 mm). The tarsus length served as a general estimate of individual size and the weight was later divided by tarsus length to obtain a measure of weight standardized by size (hereafter referred to as mass). Food intake during the period of captivity had no effect on the magnitude of the PHA-induced swelling response as revealed by the nonsignificant association between the weight change (individual weight before minus after caging) and the PHA-induced swelling response index (regression: $F_{1,43} = 1.01$, $P = 0.32$). There was no significant weight loss during captivity (paired *t* test for the weight change: $t_{44} = 1.05$, $P = 0.30$). Although some birds lost weight during the caging, this was within a 2.0 g difference (four birds lost 1.0–2.0 g, seven individuals gained 1.0–2.0 g) and as all birds were within the normal weight range at the time of release, these changes could represent normal daily individual variation. Finally, a standardized digital image of the ventral ornamented side of the bird was taken with a digital camera (see below). The bird was then ringed with a standard steel ring of the Czech ringing station (N MUSEUM PRAHA) and released. Upon release the birds did not show any signs of injury, escaping by a direct flight into the neighbouring trees. Our previous work on zebra finches, *Taeniopygia guttata*, has shown no harmful effects of the blood and biopsy sampling, with the wounds healing within a few days (M. Vinkler & H. Bainová, unpublished data). The research was approved by the Ethical Committee of the Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic and was carried out in accordance with the current laws of the Czech Republic.

Analysis of the Ornamental Coloration

For the analysis of the ornamental coloration we used standard digital photographs. As the precise description of the method adopted in this study is given in detail elsewhere (Albrecht et al. 2009), we mention it here only briefly. All photographs were taken with an Olympus C-765 digital camera (Olympus Corp., Tokyo, Japan), with fixed exposure and white balance settings, from a standard distance and under standard lighting conditions using an electronic flash in a dark tent. To enable precise standardization of the image colours we placed colour swatches (grey card GC 18 and colour & grey chart Q 14; Danes-Picta, Praha, Czech Republic) near the bird for each photograph. Photographs were analysed using Adobe PHOTOSHOP CS.3 software, version 10.0 (Adobe Systems Inc., San Jose, CA, U.S.A.). First, all images were unified in their coloration according to the white, 50% grey, 100% black and red colour on the swatch. Values of hue, saturation and brightness (HSB colour space; throughout this paper brightness is termed lightness to avoid confusion with any arbitrary colour rating; see Montgomerie 2006) were then measured at 10 points (each of area 5×5 pixels) randomly and evenly distributed over the breast colour patch. For the following analysis only average values were taken. The HSB system was adopted as it has a biologically relevant interpretation of its components. In finches, hue (with higher values in more yellowish coloration) is indicative of the type of carotenoids present in the feathers (Inouye et al. 2001); saturation reflects the quantity of carotenoid present (Saks et al. 2003a) and lightness appears to be determined by the structure of the feather's microsurface with higher values in degraded feathers (Shawkey et al. 2007). Birds are capable of recognizing reflectance in the UV part of the light spectrum (320–400 nm) and this aspect cannot be evaluated from the digital images. Nevertheless, it has been argued that, despite this fact, imaging methods can capture biologically relevant information under certain conditions (Montgomerie 2006). In our previous work we have shown that there is no reflectance in the UV part of the light spectrum in scarlet rosefinch feather ornamentation and that results obtained by digital imaging and spectrophotometry are correlated (Albrecht et al. 2009; unpublished data). Therefore the method adopted herein is suitable given the aim of the study.

Ptilochronological Assay

To measure the difference in moulting speed between males, we used ptilochronological measurements of the feather growth bars (Grubb 2006). For each individual, we took a standard digital image of the collected rectrix with a 50 mm scale, with a BenQ 5550T scanner (BenQ Corporation, Taipei, Taiwan). These digital images were then adjusted in Corel PHOTO-PAINT X3 software (Corel Corporation, Ottawa, Canada) with the function 'Local Equalization' with parameters Width 100 and Height 100, which markedly improved the visibility of the growth bars. Thereafter, the digital images were used for measurements of the total rectrix length and the mean width of the growth bars in Image Tool version 3.0 software (http://ddsdx.uthscsa.edu/dig/itdesc.html). To estimate the mean width of the growth bars, we used a segment of eight bars with the centre in two-thirds of the feather.

Histological Analysis

The tissue samples (from 15 males) were embedded in paraffin, sectioned and stained with haematoxylin and eosin (performed by BIOLAB Praha laboratory, Czech Republic; for full details see Vinkler et al. 2010a). Three sections were made from the middle part of each biopsy sample and in each of these sections three independent places were photographed (i.e. nine photographs per sample) under 40× objective magnification (microscope Olympus BX51 with digital camera Olympus DP71, Japan; acquisition software QuickPHOTO Industrial version 2.3, Promicra, Czech Republic). Later, a frame of 0.09×0.09 mm was inserted into all images in Corel PHOTO-PAINT X3 software at a random position and all cells within this frame were counted using ImageJ 1.40g software (http://rsbweb.nih.gov/ij). Thus, the cell count per 0.0729 mm^2 was obtained for the following categories of cells: lymphocytes, macrophages, heterophils, basophils, erythrocytes and thrombocytes. Two individuals had to be excluded from the analysis because of the poor quality of the sections obtained.

Statistics

Statistical analyses were performed using the statistical software R version 2.9.2 (R Development Core Team 2009). To analyse effects of hue, saturation and lightness of male feather coloration, body mass, individual size and mean growth bar width on the magnitude of the PHA swelling reaction, we used a general linear mixed-effects model with year (2006–2009) treated as a random effect. Prior to analysis, data were checked for normality using the Kolmogorov–Smirnov test. A minimum adequate model (MAM; i.e. a model with all terms significant) was obtained by backward elimination of particular terms from the full model. Candidate models were compared based on the change in deviance with an accompanied change in degrees of freedom using F statistics. The presented significances in MAMs are based on Type III sum of squares. Standard statistical tests were used otherwise. The significance level was set to $P = 0.05$.

RESULTS

The histological analysis revealed the following cellular composition of the swollen-tissue infiltrate (mean ± SE cells per 0.0729 mm^2, $N = 13$): lymphocytes: 81.54 ± 11.98; macrophages: 9.92 ± 2.33; heterophils: 46.92 ± 9.55; basophils: 184.62 ± 20.34; erythrocytes: 23.08 ± 4.91; thrombocytes: 108.92 ± 9.34. Thus, the immune response to PHA was a typical inflammation with a dominant infiltration of basophils (39.91% on average). We found a significant positive relationship between the thickness of the swelling and the number of basophils within the infiltrate, which explained more than 30% of the variability present within the data on change in patagium thickness caused by the swelling response to PHA (regression: $y = 0.002x + 0.6436$, $R^2 = 0.309$, $F_{1,11} = 4.93$, $P = 0.048$).

The effect of long-term condition on the PHA-induced inflammatory response was analysed in the complete data set ($N = 45$) with the random effect of year. Most condition-related parameters, namely male ornament saturation (Fig. 1a), male ornament lightness (Fig. 1b), individual size and mean growth bar width, had a significant influence on the change in patagium thickness caused by the swelling response to PHA. We found no significant effect of body mass or male ornament hue on the magnitude of the response (for both $P > 0.10$). The resultant MAM is summarized in Table 1. No correlation between mean growth bar width and any parameter of the ornamental coloration was found (in all cases $P > 0.5$). There was, however, a significant negative correlation between the mean growth bar width and individual size (Pearson correlation: $r = -0.33$, $t_{43} = -2.28$, $P = 0.028$).

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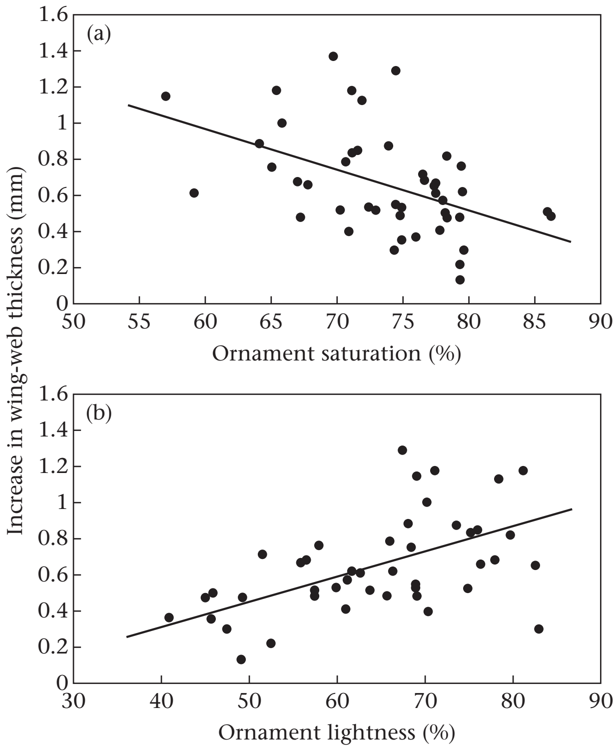

Figure 1. Association between (a) ornament saturation and (b) ornament lightness and the magnitude of the swelling response after subcutaneous PHA application.

DISCUSSION

In this study we have shown two important aspects of the PHA-induced inflammatory response in the scarlet rosefinch. First, the swelling response was (at least partly) dependent on basophil activity, as these represented the most numerous cell type within the inflamed tissue 6 h after the PHA application and there was a positive relationship between the thickness of the swelling and the quantity of basophils within the tissue. Second, the magnitude of the PHA response mirrored the long-term features of the individual, as it was significantly influenced by individual size, ornament coloration and feather growth rate at the time of moulting. However, the negative relationship between ornament saturation (referring to level of carotenoid pigmentation) and the magnitude of the response and the positive relationship between ornament lightness (referring to level of feather structure damage) and the magnitude of the response indicate that low-quality males responded more strongly than high-quality ones. Thus, our results show an opposite pattern to the usual results of the PHA skin-swelling test. Despite this fact they seem to support the prediction that superior individuals are healthier. By recruiting fewer basophils into the inflammatory site high-quality males induce a smaller swelling response. As discussed below, this could have either a mechanistic or an adaptive explanation or both.

Table 1
Minimal adequate model for the condition dependency of the magnitude of the PHA-induced immune response in scarlet rosefinch males

Fixed effects	Estimate±SE	t	P
Intercept	−3.169±1.406	−2.25	
Ornament saturation	−0.016±0.006	−2.77	0.004
Ornament lightness	0.009±0.004	2.24	0.020
Size	0.178±0.065	2.72	0.005
Mean growth bar width	0.227±0.071	3.18	0.002

$\chi^2_4 = 21.00$, $N = 45$, $P < 0.001$. Analysis is based on a mixed-effect general linear model with year (2006–2009) treated as a random effect.

The cutaneous immune response to PHA is apparently complex inflammation connected with massive infiltration of cells representing both adaptive and innate immunity (McCorkle et al. 1980; Martin et al. 2006; Vinkler et al. 2010a). This was confirmed also in the present study. Contrary to other studies reporting massive basophil infiltration only in the later phases of the response, following the heterophil influx (McCorkle et al. 1980; Martin et al. 2006) we found as early as 6 h after the PHA treatment that basophils were the predominant cell type in the tissue. This is not surprising, however, given that basophils form a major cell type in the peripheral blood even in healthy scarlet rosefinches (Vinkler et al. 2010b). Basophils represent a leukocyte type of the innate arm of immunity with crucial involvement in acute inflammatory response (Campbell & Ellis 2007). Our results therefore suggest that basophils and basophil-mediated inflammation may be of special importance in the scarlet rosefinches' immune defence. The positive relationship between the tissue basophil count and the magnitude of the PHA-induced swelling also supports the view that the primary swelling response to PHA is innate by its nature and not mainly based on cells of the adaptive arm of immunity (see also Vinkler et al. 2010a). This association, however, should be treated with some caution owing to the relatively small size of the sample of males available.

Individual size was positively associated with the magnitude of the swelling response to PHA in the scarlet rosefinch, which is a result consistent with other similar studies conducted in adult birds (see e.g. Møller & Petrie 2002). We assume that this relationship could be purely mechanistic with larger birds having larger cellular reserves to cause the inflammatory infiltration and a larger volume of skin prone to inflammation. It is unlikely that this association could result from differences in individual quality as in the scarlet rosefinch larger males do not seem to differ in quality from smaller ones (Björklund 1989). None the less, it is possible that the observed linkage might have arisen indirectly from another feature of the larger birds that remained unmeasured in our study. Although several authors also reported a relationship between the change in wing-web thickness and body mass (e.g. Gonzalez et al. 1999b; Møller & Petrie 2002; Parn et al. 2005; Mougeot 2008) we did not find any linkage of this kind. This, however, seems to be a common result in adult birds (Haussmann et al. 2005; Hõrak et al. 2007; Biard et al. 2009).

To our knowledge this is the first study to investigate the association between any ptilochronological trait and the magnitude of the PHA-induced response. Mean growth bar width is a ptilochronological marker of condition during autumn moult (Grubb 2006). The positive association between the magnitude of the PHA-induced response and the mean growth bar width is thus consistent with other observations indicating linkage of high PHA responsiveness to good condition, well-developed physical capabilities and higher survival probability (Gonzalez et al. 1999b; Soler et al. 1999; Mougeot et al. 2009). However, given the negative correlation between the mean growth bar width and the animal's size (a trait negatively related to mass-specific metabolic rate; see e.g. Furness & Speakman 2008) and the previously shown negative association between size of the individual and immature erythrocyte count (a putative marker of the haematopoiesis rate; Vinkler et al. 2010b), it is also possible that this trait is more indicative of an individual's metabolic activity during the autumn moulting period than of condition per se. In that case, the positive relationship between the PHA-induced wing-web swelling and the mean growth bar width revealed in our study would suggest that birds with a higher metabolic activity respond more strongly than less

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metabolically active birds. This relationship, nevertheless, deserves further investigation, as the interpretation of the observed association is so far, without other clues, rather speculative.

In species related to the scarlet rosefinch (such as the house finch, *Carpodacus mexicanus*) males with more elaborate ornaments cope better with infectious pathogen diseases (Hill & Farmer 2005). Carotenoid-based ornamental coloration is composed of three independent variables: hue (indicating mostly the type of pigments deposited), saturation (indicating amount of carotenoids deposited with higher values in healthier males) and lightness (referring to surface structure with higher values indicative of feather damage; Inouye et al. 2001; Hill 2002; Saks et al. 2003a; Shawkey et al. 2007). Our results seem to contradict most of the current evidence on the association between immunity or health and ornamentation as they show a negative linkage of the magnitude of the PHA-triggered immune response to plumage saturation and a positive one to feather lightness. In male blackbirds, *Turdus merula* (Faivre et al. 2003b), zebra finches (Blount et al. 2003; McGraw & Ardia 2003), red grouse, *Lagopus lagopus scoticus* (Mougeot 2008), red-legged partridges, *Alectoris rufa* (Perez-Rodriguez et al. 2008; Mougeot et al. 2009), moorhens, *Gallinula chloropus* (Fenoglio et al. 2004) and ostriches, *Struthio camelus* (Bonato et al. 2009) beak, comb and skin carotenoid-based coloration is often positively associated with PHA responsiveness. Nevertheless, in contrast to this evidence, another study on carotenoid-based bill coloration in male blackbirds found no association of this kind (Biard et al. 2009). In addition, Navara & Hill (2003) and Saks et al. (2003b) did not find any positive linkage between the carotenoid-pigmented plumage coloration and PHA responsiveness in American goldfinches, *Carduelis tristis*, and greenfinches, *Carduelis chloris*, respectively. Even a negative relationship has been reported between the PHA response and some other components of male ornamentation (in peafowl, *Pavo cristatus*, the diameter of the train ocelli, Møller & Petrie 2002, and in common yellowthroats, *Geothlypis trichas*, the black mask size, Garvin et al. 2008). Gonzalez et al. (1999a) also showed that, in house sparrows, *Passer domesticus*, the relationship between the magnitude of the PHA-induced response and melanin-based ornamentation varies from positive to negative in the course of a year. This might be caused by changes in the immunological mechanism of the response. However, as carotenoid-based coloration is an individually consistent trait (Perez-Rodriguez 2008), we may expect that in the long term PHA responsiveness would also be individually consistent.

We believe that the lack of agreement between studies on different species may lie in the immunological background of the response. The results of the PHA skin-swelling test are often interpreted as 'T-cell-mediated immunocompetence' (e.g. Tella et al. 2008; Hawley et al. 2009; Kilgas et al. 2010). Much of the evidence currently available nevertheless suggests that this interpretation is problematic (McCorkle et al. 1980; Martin et al. 2006; Sarv & Hõrak 2009; Vinkler et al. 2010a; Sepp et al. 2011; see also Saks et al. 2006; Kennedy & Nager 2006; Owen & Clayton 2007). On the contrary, it seems that there is no clear evidence of major involvement of T cells in the primary inflammatory immune response to PHA (see Vinkler et al. 2010a). Also, in this study we have shown major involvement of basophils in the swelling response, which indicates the innate-immunity and not adaptive-immunity nature of the reaction. Innate immunity and adaptive immunity are distinct in their mechanisms and parasite-killing potential as well as in ontogeny, energetic costs and evolutionary trade-offs (see e.g. Hasselquist 2007; Lee et al. 2008; Palacios et al. 2009). If innate immunity is involved it is much more difficult to assume that higher responsiveness to PHA is a 'good' trait. The stronger response may result, for instance, from currently activated inflammatory processes elsewhere in the organism. As emphasized in several recent articles, the magnitude of an immunological response does not necessarily mean better parasite resistance or even better lifetime fitness (see e.g. Owen & Clayton 2007; Graham et al. 2011). On the contrary, greater magnitude of an immune response may even indicate ineffective antiparasite protection or immunopathology (Graham et al. 2005). This can be seen also in the results of immunoecological studies. For instance, in male blue peafowl Møller & Petrie (2002) found a positive association between the PHA response and the H/L ratio, a trait known to be associated with stress and disease (Ots et al. 1998; Davis et al. 2008). In several passerine species individuals harbouring more severe parasite infections produce stronger cutaneous responses to PHA application (Christe et al. 2000; Gwinner et al. 2000; Saks et al. 2006). This is consistent with our explanation that, at least in some cases, the magnitude of the PHA response correlates negatively with the individual's current state of health. This could be for purely mechanistic reasons, perhaps as a result of elevated pretreatment blood levels of cells involved in inflammation (e.g. basophils or heterophils) or plasma-mediated proinflammatory cytokines. The scarlet rosefinch has an unusual haematological profile (Vinkler et al. 2010b) which is different to that of most other passerines and this might be responsible for the association observed in this study.

Our results may also be viewed adaptively. High-quality, highly ornamented males might invest less in their inflammatory immune responsiveness. Inflammation is a destructive process and if other, less harmful, immunological mechanisms are available, it need not be the preferred one. Moreover, according to the immunocompetence handicap hypothesis, hormonal regulation of an ornament's expression might present a trade-off between ornamentation and immunity (Folstad & Karter 1992). A similar situation could also be predicted by the carotenoid maintenance hypothesis (Vinkler & Albrecht 2010). None the less, it is uncertain whether a trade-off of this kind would also suppress immune responses in the prebreeding season when there is no ornament expression. As birds may also differ in the dynamics of the cutaneous immune response to PHA (Biard et al. 2009), it is also possible that the difference between high-quality and low-quality individuals may be in the time curve of the swelling response. Most of these hypotheses are not mutually exclusive. Our results, however, do not allow for the investigation of temporal dynamics of the response or its hormonal background and, hence, leave these possibilities untested.

To conclude, although carotenoid-pigmented living tissues (i.e. dynamic traits such as beak rhamphotheca or skin) are most probably better indicators of the current physiological state than feathers (Faivre et al. 2003a), feather-based ornaments might better reflect long-term individual qualities. Although short-term changes in health might influence the individual responsiveness to PHA, our results suggest some general long-term consistency of the individual proinflammatory potential. In the scarlet rosefinch we found a negative association between the magnitude of the PHA-induced skin-swelling response and the feather-based ornamental coloration suggesting that inferior individuals respond more strongly than the high-quality ones. This result is meaningful given the known immunological nature of the response in this species. The low-quality individuals mobilize more basophils into the inflamed tissue, possibly owing to generally higher levels of these leukocytes in their bodies. Our results hence suggest a positive relationship between ornamentation and health in rosefinch males. The greater magnitude of the response indicates not higher quality in the scarlet rosefinch but on the contrary, poorer health. None the less, this might be the case only in some species as the magnitude of the PHA-induced response clearly depends on the overall immunology of the species in question. A different relationship between the magnitude of the PHA-induced response and individual quality may be expected, for instance, in species with lower numbers of circulating proinflammatory cells. To obtain any conclusions to this topic further studies in more species are needed. As basophils seem to be crucial for the inflammation at least in some passerine species, further research should also be focused on deeper investigation of immunological activity of passerine basophils.

Acknowledgments

We are grateful to Martin Lundák, Lubor Červa, Jaroslav Jelínek, František Zicha, Pavel Jaška, Hana Mrkvičková, Radka Poláková and Marta Promerová for their help with the field work and we acknowledge Zdena Csiebreiová for her technical assistance in the laboratory. Our gratitude belongs last but not least to Dagmar Vinklerová, Jitka Poplová, three anonymous referees, the Editor Sergio Castellano and Managing Editor Angela K. Turner for their valuable comments on the manuscript. This work was supported by the Grant Agency of Charles University in Prague (project 191/2004/B-Bio), the Grant Agency of the Academy of Sciences of the Czech Republic (project IAA600930608) and the Czech Science Foundation (projects 206/06/0851 and P505/10/1871). T.A. was partially supported by the Research Centrum project No. LC06073.

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