

The effect of immune system state and function on the sexual selection in birds

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Charles University in Prague, Faculty of Science

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on the sexual selection in birds**

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Michal Vinkler
Prague, 30th May 2011

Title

The effect of immune system state and function on the sexual selection in birds

Title in Czech

Vliv stavu a funkce imunitního systému na pohlavní výběr u ptáků

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To my parents whose chary care made me one of the oldest altricial young ones in the animal kingdom and thus enabled the formation of this thesis.

To my friends and colleagues without whom this work would be hardly possible.

To my grandmother for baking the best cakes and sweets I have ever eaten.

PREFACE

Modern scientific research is inconceivable without cooperation of a number of people, often from several different institutions. This is also true for the investigation summarized in this thesis in which help of many people made the work possible.

First of all, I am highly obliged to my supervisor Tomáš Albrecht for his wide-ranging support and in particular for his initial idea of studying the involvement of immune system in the sexual selection in birds. The evolution of immunity is one of the most fascinating issues in biology I have had the pleasure to come across. I hope this thesis is not the end of the research started in this field but rather its beginning. Tomáš built for me and other students of his a stable bridge between the Charles University in Prague and the laboratories of the Institute of Vertebrate Biology of the Academy of Sciences of the Czech Republic located in Studenec – an unification that was vitally important for many aspects of the work presented in this thesis. He has been always opened to new ideas and never discouraged me from any novel directions of my research though those were sometimes only distantly related to our original plans. My great thanks also belong to Pavel Munclinger who was my co-supervisor during my pre-graduate studies and later a good senior colleague of mine. For a long time Pavel kindly provided me except of a desk in his office also with his friendly help and practical advice on my work.

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I am particularly obliged to several people for enabling me as a zoologist to infiltrate into the field of immunology. Vladimír Holáň graciously provided me lots of advice and many suggestions since the very beginnings of this work (though I could never be sure whether some of the suggestions he made were meant real or as a joke). I hope I will have the possibility to pay his help back; at least in my support of his nestling-ringing effort. I am also very grateful to Pete Kaiser for opening some brand-new prospects for further research for me. Although the three-week stay in Pete's lab in Compton was not long enough to let me learn everything important I would wish to, Pete, Lisa Rothwell and Zhiguang Wu kindly provided me with as-many-as-possible useful instructions I follow. Furthermore, I am thankful to Pavel Hyršl, Petr Šíma, Josef Geryk and Dana Průková for their aid with immunological tasks and to Ivo Konopásek and Jana Beranová for helping me with my attempts in microbiological studies that were, however, never crowned with success.

It is my pleasure to express my gratitude to all people who advised me and helped with the preparation of the manuscripts of the articles included in this thesis. I received many useful comments especially from Lukáš Kratochvíl, Jan Votýpka, Martina Pokorná, Miroslav Šálek, Jakub Kreisinger, Drahoslav Pravda, Stuart Baird, Joëlle Goüy de Bellocq, Edwin L. Cooper and Petr Šíma. Last but not least my great thanks belong to Dagmar Vinklerová, Mark Gibson, Heidi C. Hauffe, Alison Golinski and Marek Vinkler who kindly devoted much of their time to the language corrections.

Finally, I would like to thank my family and my helpmeet Jitka for all the care and support I received from them in the time of my work on this thesis.

Looking backwards, at least in my own personal opinion, some interesting pieces of work have been done by our group in the research of the Parasite-mediated sexual selection. However, at the same time I also feel much shame about the fact that even more of our plans have never been realised and quite a bulkily amount of material and data still (in some cases for many years) wait in drawers and freezing boxes for their revival. I, therefore, hope that I will maintain the favour of all my colleagues and collaborators also in the future and that there will be a possibility for us to finish most of the work started.

Michal Vinkler
Prague, 27th May 2011

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SUMMARY

Evolution is a most fascinating feature of all living things in our world. The means of organismal evolution are multiple and diverse, comprising mainly of two categories of processes: natural and sexual selection. Both of these broad ranges of phenomena may contribute to evolutionary adaptations in health and disease. The importance of natural selection for evolution of anti-parasite resistance is known and commonly accepted for more than half a century. What is less often assumed and discussed is the role of sexual selection in evolution of the host immunity. The questions of immunity impact on the process of sexual selection and sexual selection for increased anti-parasite resistance are wide issues of the present research in natural sciences. Their clarification requires multidisciplinary-based investigation combining a variety of partial results into a single united paradigm. Although I have chosen for my thesis only a relatively small part of this extensive topic: the signalling function of carotenoid-pigmented ornamental traits in birds, it was far beyond my possibilities to cover it in all its complexity and in detail. Therefore, my co-workers and I have chosen model species (Scarlet rosefinch, *Carpodacus erythrinus* and Zebra finch, *Taeniopygia guttata*) to investigate and several specific issues to study, in order to fill some of the important gaps of the current knowledge. The result of our four-year endeavour is compiled in this thesis. In the field of the general theoretical frameworks we aimed to contribute to the current idea of the mechanism preserving honesty in the health signalling through carotenoid-based ornamental traits. Throughout proposing the Carotenoid maintenance handicap hypothesis we pointed out that optimisation instead of maximisation of the carotenoid intake and ornamental display may be physiologically convenient for the individual. In the Scarlet rosefinch we confirmed the role of the carotenoid-based ornamental colouration in the process of sexual selection and investigated several partial aspects of the association between immunogenetics, immune function, health and the ornament expression and mate choice. In the Zebra finch we have shown the present way of interpretation of results of a commonly used immunoecological test for cellular immune response assessment (the phytohaemagglutinin skin-swelling test) to be erroneous and proposed a corrected view on the immunological mechanisms behind the test. Moreover, in a separate contribution we highlighted that the current ecological terminology using general 'immunocompetence' as a value for measuring the anti-parasite resistance is far too simplistic and should be replaced by more exact descriptions of the immune responsiveness. Finally, we have attempted to propose a novel direction of investigation in the wildlife immunogenetics starting with the description of Zebra finch Toll-like receptor 4 as a model molecule of an innate immunity receptor essential for antibacterial defence. As this receptor regulates the intensity of inflammation (a process known to influence the redox balance and antioxidative protection of tissues), we believe this is a good starting point for further research in the linkage between the carotenoid-based ornamentation and immunity.

SOUHRN

Evoluce je jedním z nejzajímavějších dějů v živém světě. Ačkoliv využívá nejrůznějších cest, v principu sestává ze dvou hlavních procesů: přírodního a pohlavního výběru. Obě tyto široké kategorie jevů mohou přispívat k evolučním adaptacím v oblasti zdraví a nemoci. Zatímco význam přírodního výběru je v tomto směru obecně znám a přijímán po více než půl století, role pohlavního výběru v evoluci imunity je zřejmá méně, a tak je také méně často diskutována. Přesto patří otázky týkající se vlivu imunity na proces pohlavního výběru a role pohlavního výběru v evoluci zvyšování rezistence proti parazitům mezi zásadní témata současného výzkumu v biologických vědách. Jejich zodpovězení vyžaduje multidisciplinární přístup kombinující nejrůznější poznatky do jednotného paradigmatu. Přestože jsem si pro svou disertační práci vybral jen relativně malou část tohoto širokého tématu (signální funkce karotenoidních ornamentů u ptáků), bylo nad rámec mých možností pokrýt studovanou problematiku v celé její komplexnosti a hloubce. Proto jsme se s mými spolupracovníky pokusili zvolit vhodné modelové druhy výzkumu (hýl rudý, *Carpodacus erythrinus* a zebříčka pestrá, *Taeniopygia guttata*) a několik vybraných okruhů výzkumných témat tak, abychom zaplnili některé významné mezery v současném poznání v této oblasti. Výsledek našeho čtyřletého úsilí je shrnut v této disertační práci. Na poli teoretických studií jsme se snažili přispět k pochopení mechanismu udržujícího čestnost v signalizaci zdravotního stavu jedince prostřednictvím karotenoidních ornamentů. V námi navrhované hypotéze handicapu v důsledku udržování vysoké hladiny karotenoidů (Carotenoid maintenance handicap hypothesis) jsme vyzdvihli význam optimalizace namísto maximalizace jak v příjmu karotenoidů, tak i následné tvorbě karotenoidního ornamentu pro celkovou fyziologii jedince. U hýla rudého jsme potvrdili vliv karotenoidního ornamentálního zbarvení na pohlavní výběr a prozkoumali některé dílčí aspekty vztahu mezi imunogenetikou, aktivitou imunitního systému, zdravotním stavem, tvorbou ornamentace a výběrem partnera. Na příkladu zebříčky pestré jsme ukázali, že současná interpretace výsledků některých testů používaných v imunoekologii pro stanovení buněčné imunity (fytohemaglutininový kožní test) je chybná a navrhli jsme správnější pohled na imunologické mechanismy v pozadí tohoto testu. Krom toho jsme v samostatném příspěvku poukázali na skutečnost, že nyní používaná ekologická terminologie využívající obecnou „imunokompetenci“ jako měřítko anti-parazitární rezistence je příliš simplistická a měla by být nahrazena přesnějším popisem imunologické odpovědi. Konečně v oblasti imunogenetiky jsme se pokusili navrhnout nový směr bádání u volně žijících zvířat a začali jsme popisem Toll-like receptoru 4 u zebříčky pestré jako modelové molekuly receptoru vrozené imunity nezbytného v obraně proti bakteriálním infekcím. Jelikož je tento receptor zapojen do regulace intenzity zánětlivé odpovědi, věříme, že se jedná o vhodný výchozí bod pro výzkum vztahu mezi karotenoidními ornamenty a imunitou.

LIST OF PAPERS

This thesis consists of the following ten papers (one book chapter in press, seven published articles and two submitted manuscripts being under the review at the time of printing this thesis) that are referred in the text of the thesis by the Roman numerals given below:

Book chapter in press

- I. **Vinkler M.**, Svobodová J., Maršík P. & Albrecht T.: Carotenoids and health signalling in animals. In: Yamaguchi M. (ed) Carotenoids: Properties, Effects and Diseases, Nova Science Publishers, Hauppauge, NY, USA; ISBN: 978-1-61209-713-8; scheduled to be published in 2011

Published articles

- II. **Vinkler M.** & Albrecht T. (2010): Carotenoid maintenance handicap and the physiology of carotenoid-based signalisation of health. *Naturwissenschaften* 97:19-28; DOI: 10.1007/s00114-009-0595-9
- III. **Vinkler M.**, Bainová H. & Albrecht T. (2010): Functional analysis of the skin-swelling response to phytohaemagglutinin. *Functional Ecology* 24: 1081-1086; DOI: 10.1111/j.1365-2435.2010.01711.x
- IV. Albrecht T., **Vinkler M.**, Schnitzer J., Poláková R., Munclinger P. & Bryja J. (2009): Extra-pair fertilizations contribute to selection on secondary male ornamentation in a socially monogamous passerine. *Journal of Evolutionary Biology* 22 (10): 2020-2030; DOI: 10.1111/j.1420-9101.2009.01815.x
- V. **Vinkler M.**, Schnitzer J., Munclinger P., Votýpka J. & Albrecht T. (2010): Haematological health assessment in a passerine with extremely high proportion of basophils in peripheral blood. *Journal of Ornithology* 151 (4): 841–849; DOI: 10.1007/s10336-010-0521-0
- VI. Promerová M., **Vinkler M.**, Bryja J., Poláková R., Schnitzer J., Munclinger P. & Albrecht T. (2011): Occurrence of extra-pair paternity is connected to social male's MHC-variability in the scarlet rosefinch *Carpodacus erythrinus*. *Journal of Avian Biology* 42: 5-10; DOI: 10.1111/j.1600-048X.2010.05221.x
- VII. **Vinkler M.** & Albrecht T. (2009): The question waiting to be asked: Innate immunity receptors in the perspective of zoological research. *Folia Zoologica* 58 (Suppl. 1): 15–28
- VIII. **Vinkler M.**, Bryjová A., Albrecht T. & Bryja J. (2009): Identification of the first Toll-like receptor gene in passerine birds: TLR4 orthologue in Zebra finch (*Taeniopygia guttata*). *Tissue Antigens* 74: 32-41; DOI: 10.1111/j.1399-0039.2009.01273.x

Submitted articles

- IX. **Vinkler M.** & Albrecht T.: Handling 'immunocompetence' in ornithological studies: Do we operate with confused terms? (submitted to *Journal of Avian Biology* in April 2011).
- X. **Vinkler M.**, Schnitzer J., Munclinger P. & Albrecht T.: Phytohaemagglutinin skin-swelling test in scarlet rosefinch males: low-quality birds respond stronger (submitted to *Animal Behaviour* in May 2011).

GENERAL INTRODUCTION

Throughout the world that we inhabit parasitism is one of the most common food-acquisition strategies. Virtually any free-living animal species hosts some parasites and in most large taxa of organisms at least some species adopted the parasitic way of life. In their relationship to their host parasites always decrease the host fitness by reducing its nutrition acquisition and occasionally also by causing pathogenesis (Clayton & Moore 1997). In animals the immune system forms a basic tool serving them to protect their organisms from parasite attacks. The immune system function has therefore a paramount importance for animal survival and reproduction, i.e. for the two essential features governing the lifetime fitness of each individual. Although much of the immune system evolution is undoubtedly realised by the means of natural selection, sexual selection may importantly contribute to microevolutionary processes taking place at the species- or population-level (see, e.g., Hamilton & Zuk 1982; Milinski 2006). This provides us with the answer to the first and most important question that may be posed to the topic of this thesis – the question “*Why?*” Why should we expect any effect of the immune system state or function on the course of sexual selection? Why is this important? Despite reasonable research effort over the past two decades in resolving several issues of this topic, our comprehension of the links between sexual selection and evolution of immunity is still only limited. The research covered in this thesis aimed to contribute to the knowledge presently available in animal ecological and evolutionary immunology.

What we observe in nature is a sexual selection that is taking place in a great variety of different ways and forms (Andersson 1994). It is thus probable that the evolutionary outcome of the selection will slightly or even substantially differ from species to species. However, several basic modes of sexual selection can be identified. The mate choice may be either male- or female-based. We can expect that the choosiness of each of the sexes will be dependent on their possible gains from the mate choice. Another division can be made according to the clue for selection among potential mates such as e.g. a mate choice based on the olfactory signals, optical signals, direct physical contact and others. It can be predicted that different signals contain distinct information on the sexual partner which may be important for realisation of an appropriate mate choice. These natural modes of sexual selection may be used to search for some general evolutionary trends and principles valid across species. For the purposes of this thesis I decided to focus only on a small subset of all these modes and dedicated the research to studying the effect of female selection for carotenoid-based ornamental traits in birds. In carotenoid-based ornamental colouration the best evidence linking health state with sexual selection has been gathered in the previous research (see, e.g., Møller et al. 2000; Hõrak & Saks 2003; Peters 2007). The general introduction to evolution of the carotenoid-based traits has been summarised as an individual review paper included in this thesis – a book chapter which is going to be published in Yamaguchi M. (ed) *Carotenoids: Properties, Effects and Diseases*, Nova Science Publishers, Hauppauge, NY, USA [please see Paper I; Vinkler M., Svobodová J., Maršík P. & Albrecht T.: *Carotenoids and health signalling in animals*; scheduled to be published in 2011].

At the current state of knowledge it seems to be well supported that selection for carotenoid-based ornamental traits may contribute to evolution of anti-parasite resistance in the manner predicted by Hamilton & Zuk (1982). Although the physiological process responsible for the relationship between ornamentation and health remains unresolved, the existing theoretical models (e.g. Lozano 1994; von Schantz et al. 1999; Peters 2007; Paper II) propose at least some idea about the mechanisms by which the selection for higher anti-parasite resistance could be realised. This provides us with the answer to

another essential question related to the topic of this thesis, i.e. the question “*How?*” How is the sexual selection realised to maintain or even improve the anti-parasite resistance in the offspring? Nonetheless, a third and equally important question is still remaining. The question stands “*What?*” What are the anti-parasite resistance genes selected in animal sexual selection? Undoubtedly these are the genes related to the immune function but we have no idea which of these genes precisely. Immunity of animals relies on a great number of genes and many of these are vitally important to the appropriate development, growth and reproduction (Davison et al. 2008). Others have their specific roles only in host-parasite interactions. Can all of these genes become a subject of sexual selection equally well? This question is presently perhaps much more puzzling than the others as, as far as I know, there is so far no general theoretical framework to this topic and the evidence available is even more scarce than the one to the other issues. In vertebrate ecological and evolutionary immunology a great attention has been paid mainly to components of the adaptive arm of immunity. Given the relationship which has been found between the diversity in genes of the Major Histocompatibility Complex (MHC) and mate choice in mice and fish (Penn 2002; Milinski 2006) the majority of the ecologically based research focused on these genes. However, as pointed out by Acevedo-Whitehouse & Cunningham (2006), MHC is not enough to understand the animal immunogenetics and other genes should be also investigated. In particular, variability in genes encoding molecules that directly interact with parasite-borne structures may be responsible for differences in the host resistance and thus could be also subjected to the parasite-mediated sexual selection. Among these, especially Pattern recognition receptors (PRRs) may be important as they represent one of the first means of the immune system to detect the parasite and to appropriately direct both the innate and adaptive immune responses. A general introduction to the possible role of PRR polymorphism in the host evolution of anti-parasite resistance is given as an individual review paper included in this thesis [please see Paper VII].

Answering the three basic questions, i.e. “*Why?*”, “*How?*” and “*What?*” in host-parasite interactions is the main task of ecological and evolutionary immunology (also known as ecoimmunology or immunology). In this thesis I aimed to contribute to the resolving of these three questions with special focus on the Scarlet rosefinch as a model passerine species.

AIMS OF THE THESIS

Previous research in carotenoid-based colouration in animals suggested its signalling function in sexual selection with respect to individual health (e.g. Hamilton & Zuk 1982; Milinski & Bakker 1990; Møller et al. 2000; Hill 2002). In the work included in this thesis my collaborators and I aimed to investigate the evolutionary implications of the relationship between health and male ornamental colouration in a novel model species – the Scarlet rosefinch (with specific metodological tasks being solved in the Zebra finch). The specific aims of the research were as follows:

- To elaborate the theoretical apparatus to the sexual selection for anti-parasite resistance and mechanism preserving honesty of the health signalling through carotenoid-based ornamentation.
- To improve the knowledge of the immunological background of the methods used for immunity assessment in the ecological studies.
- To investigate the role of the male carotenoid-based ornamental colouration in mate choice in the Scarlet rosefinch.
- To examine the relationship between health and ornamentation in this species.
- To investigate the genetic basis of the difference in immunity in birds.



Scarlet rosefinch male (photo J. Schnitzer)

GENERAL METHODS

Study species

The Scarlet rosefinch *Carpodacus erythrinus* (Pallas, 1770) is a small sized (ca 20 g) sexually dichromatic passerine belonging to the subfamily Carduelinae (Cramp et al. 1994). Females and immature males are predominantly olive-brown while the adult male plumage is coloured in tones of red on the chest, crown and rump. These colourful feather traits are pigmented by carotenoids (Maršík et al. unpublished data). Males vary greatly with respect to the colouration of their ornaments: the plumage colour ranges from bright red to drab reddish and even orange. The phenomenon of delayed plumage maturation occurs in this species (Jenni & Winkler 1994), i.e. males acquire the ornamental plumage of adults as late as during the moulting in their second year of life while they may be sexually active as soon as in their first year. This enables the differentiation of young males from the elder ones. The area of distribution of the Scarlet rosefinch covers mainly the temperate continental zone of the Palaearctic region. Of the several recognised subspecies only *C. e. erythrinus* occurs in Europe (Cramp et al. 1994). The species represents a long-distance migrant with wintering grounds located in the South Asian region, probably particularly in India (Cramp et al. 1994). Scarlet rosefinch breeding is being reported in the Czech Republic since the second half of the 20th century and despite the presently growing population of the species in this country the species is relatively rare with only a local distribution (the breeding population is estimated to 1300-2000 pairs; Štastný et al. 2006). The food of the rosefinches includes mainly seeds, buds and some invertebrates. Contrary to most other passerines even nestlings are fed from a great part with seeds rather than with insects (Cramp et al. 1994).

The extremely long migration causes that Scarlet rosefinches spend only a very short time (from May to mid July) on their breeding sites in Europe. In the Czech Republic the breeding season of this species starts in the third decade of May. Males are supposed to be the first to appear on the breeding grounds and in a few days they are followed by females (Björklund 1990). After arrival the males start to attract females by song with display and the pairs are formed as soon as individual females reach the grounds (Cramp et al. 1994). Males do not defend any territory before nesting (Björklund 1990). The Scarlet rosefinch represents a socially monogamous species with a relatively large proportion of extra-pair paternities in broods (Albrecht et al. 2007). Pairs breed only once per year, with one replacement clutch following failure (Albrecht 2004). In our study population the nests are placed mainly inside the spiraea shrubs. Females lay usually 4-6 bluish-green eggs in the clutch (Cramp et al. 1994). Incubation takes around 11-12 days and the offspring stays in the nest up to 10 days of age. Then the fledglings leave the nest being usually still flightless and are fed by their parents for another 2 weeks. In our population the nest survivorship ranged between 41% and 83% (Albrecht 2004). Although the Scarlet rosefinch is related to the American House finch (*Carpodacus mexicanus*), a well known model for studies in ornamental function in sexual selection in birds, the biology of the two species is remarkably different, with the House finch being sedentary and with only minor proportion of the extra-pair paternities (Hill 2002; Lindstedt et al. 2007).

The Zebra finch *Taeniopygia guttata* (Vieillot, 1817) is a small (ca 15 g) sexually dichromatic passerine belonging to the subfamily Estrildinae (Zann 1996). This species is naturally widely distributed throughout the continental Australia as well as the islands of the Lesser Sundas archipelago inhabiting a variety of habitats from arid areas to woodlands. The captive breeds were established in Europe in the second

half of 19th century and since 1920s almost no new birds entered the captive population. Thus, the birds currently utilised in the laboratory experiments are fully domesticated (Zann 1996). Despite a variety of colour morphs available no standard laboratory inbred lines were created. Thus in most of the research the hobby bred birds are being used. Zebra finch belongs presently among three avian species with the known full-genome sequence (<http://www.ensembl.org>).

Study site and conditions of the field work

The research in the Scarlet rosefinch was carried out on a population of the this species breeding in the Vltava river valley, Šumava National Park, Czech Republic (N 48°48'–48°50', E 13°55'–13°57', ~730 m above sea level). The habitat represents several isolated patches of a wet shrubby meadow (the total area of the meadow study sites is about 200 ha) surrounded by a rural landscape from one side and hills covered with forest from the other. All study sites were very similar in their plant composition. Shrubs represented mainly by spiraea (*Spiraea salicifolia*) and willows (*Salix* spp.) covered 10–20% of the total breeding-habitat area. The herbaceous layer consisted mainly of meadowsweet (*Filipendula ulmaria*), sedge (*Carex* spp.), and marsh grasses (*Phalaroides arundinacea*, *Glyceria maxima*). The patches are regularly flooded during the April–May period and water persists locally in channels and pools till the beginning of July (Albrecht 2004). The field work covered the pre-breeding and breeding period of rosefinches (from the second half of May to early July) in nine study seasons 2000–2008. My personal involvement in the field research comprised the seasons 2004–2008. However some of the samples collected in the previous years were also co-included into this thesis. In each year the adult birds were caught into mist-nets before the breeding season and later at the nest sites. In the time of egg lying and incubation the whole area of the study site was searched for the rosefinch nests. Typically 15–20 nests were found and included into the research. For details to the methodology of the individual studies please see the methods given in the separate papers.

Haematological and histological methods

Part of the research performed in rosefinches and Zebra finches involved haematological and histological methods. The approaches adopted represent examinations common in veterinary practice (Campbell & Ellis 2007). We estimated the leukocyte frequencies in blood or skin tissue samples, the absolute counts of peripheral blood leukocytes and erythrocytes and the haematocrit. The blood or tissue samples collected were minimal to provide evaluable data and the treatments were never dangerous to the health or life of the animal. The blood-borne cells and tissue based leukocytes were identified based on the avian haematological and cytological atlases by Lucas & Jamroz (1961) and Campbell & Ellis (2007). The leukocyte counts in blood and tissues were previously shown to be valuable indicators of health, physiological stress and inflammation in birds (Ots et al. 1998; Martin et al. 2006; Davis et al. 2008).

Measuring inflammation

Measuring immune responsiveness (often synonymised with 'immunocompetence') in free-living birds is problematic for several reasons. Firstly, field work often lacks the possibility of immediate usage of the laboratory equipment required for processing of the living samples for immunological assays; secondly, the samples that are possible to be collected are too small in their volume to enable most of the standard assays and finally (and most importantly), the molecular tools developed for the evaluation

of the immune function in laboratory animals simply do not exist for other, non-model, species so far. Nevertheless, several relatively simple approaches have received wider popularity in immunoeological studies. The phytohaemagglutinin (PHA) skin-swelling test also belongs to this class (Kennedy & Nager 2006; Ardia & Schat 2008). This test has been used in the past two decades for measuring the cell-mediated immune responsiveness both in avian (from the more recent reports see, e.g., Gasparini et al. 2009; Hawley et al. 2009; Moreno-Rueda 2010; Kilgas et al. 2010; Tobler et al. 2010; Griggio et al. 2010) and mammalian (see, e.g., Hernandez et al. 2005; de Bellocq et al. 2007; Fernandez-De-Mera et al. 2009; Allen et al. 2009) studies.

The test is based on subcutaneous application of PHA which results in a remarkable swelling of the tissue that can be metrically measured. PHA is a plant lectin produced by *Phaseolus vulgaris* and known for its T-cell-mitogenic potential (Nowell 1960; Rigas & Johnson 1964; Majumdar et al. 1990; Licastro et al. 1993). The PHA molecule is a tetrameric structure consisting of combinations of two basic subunits with either lymphocyte-specific or erythrocyte-specific binding activities (Yachnin & Svenson 1972; Leavitt et al. 1977; Felsted et al. 1977). The L-(lymphocyte-binding-)subunits were shown to form high affinity bonds with the α/β heterodimer or γ chains of the T-cell receptors (TCRs) triggering the signalisation cascade of TCR irrespectively to its own specificity and forming thus signal for the cell activation and proliferation (Chilson & Kelly-Chilson 1989). The E-(erythrocyte-binding-)subunit causes haemagglutination (Rigas & Osgood 1955). These two subunits may form a mixture (PHA-P) of 5 isolectins differing significantly in their biological properties.

In birds the *in vivo* PHA skin-swelling test is performed in the wing-web (patagium) site and evaluated in the form of a swelling index. The original procedure calculated the swelling index as the difference in the magnitude of the tissue between the PHA-treated and saline-buffer-treated (control) wing of the bird (see, e.g., Lochmiller et al. 1993). This protocol was simplified by Smits et al. (1999) who proposed calculation of the swelling index as the difference in the wing-web thickness prior and after the swelling response. Several authors performed a sensitising injection of the PHA before the treatment one (Johnsen et al. 2000; Kleven et al. 2006). The test is often assumed to reveal a 'T-cell mediated immunocompetence'. The *in vivo* response to PHA was shown to be thymus-dependent (Goto et al. 1978) but any direct evidence supporting the idea that the swelling is caused by proliferation or infiltration of the T cells is missing. The cellular dynamics of the response was described by Goto et al. (1978) and McCorkle et al. (1980) in chicken (*Gallus gallus f. domestica*) and more recently by Martin et al. (2006) in house sparrow (*Passer domesticus*). It seems that the swelling is caused by inflammation associated with infiltration of macrophages and heterophilic granulocytes shortly after the application of PHA which is followed by increased infiltration of eosinophils, lymphocytes and especially basophils within next 24 hours and cannot be interpreted as a T-cell-mediated response [for our results to this topic and further discussion please see Paper III of this thesis]. 48 hours after the treatment the reaction is usually already noticeably decreasing. Contrary to the dynamics of the cellular infiltration the total magnitude of the swelling seems to be invariable within the interval between 6 and 24 hours after the administration of PHA into the tissue and there is a positive correlation between several measurements in time (Navarro et al. 2003; Møller et al. 2003). The PHA treatment does not represent a great source of stress to the studied animals (Merino et al. 1999; Hōrak et al. 2000). Nonetheless, there are several methodological aspects that have to be taken into account when using the method for inter-individual comparisons. Firstly, it is impossible to repeat the test in the same animal for several times as any repeated application of the PHA co-stimulates the memory cells in the adaptive immune response (Tella et al. 2008), thus causing a response different

by its nature from the first one. Secondly, the intensity of the PHA swelling reaction may be modulated by stress prior the treatment (Lazarevic et al. 2000; Ewenson et al. 2003; Viswanathan et al. 2005; Morales et al. 2006) as well as by temperature or food abundance (Liffield et al. 2002). That is why it is important to keep all the examined individuals under the same conditions. Thirdly, the response may also vary between sexes (Cheng & Lamont 1988; McGraw & Ardia 2005) and age classes (Lavoie et al. 2007) or fluctuate in time (Navarro et al. 2003; Møller et al. 2003; Bourgeon et al. 2006; Martínez-Padilla 2006).

Molecular methods

Several methodical approaches of molecular genetics were applied in this thesis. Some of these were partially performed by me, others by my co-workers. These approaches included standard DNA and RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR), genomic DNA and complementary DNA PCR, cloning, sequencing, 3'RACE and 5'RACE (Rapid Amplification of cDNA Ends) reactions and single-strand conformation polymorphism (SSCP) analysis. The analysis of the data obtained was performed using several bioinformatic tools including the GenBank BLAST (<http://www.ncbi.nlm.nih.gov/>), BioEdit Sequence Alignment Editor with implemented ClustalW tool (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>), SeqScape version 2.5 (Applied Biosystems), GeneMapper version 3.7 (Applied Biosystems), EXPASY Translate tool (<http://www.expasy.ch/>), Simple Modular Architecture Research Tool (SMART) version 5.0 (<http://smart.emblheidelberg.de/>), Molecular Evolutionary Genetic Analysis (MEGA) software version 4.0 (<http://www.megasoftware.net/>), HYPHY software (<http://www.datamonkey.org>) and 'IR macroN3' (<http://www.zoo.cam.ac.uk/zoostaff/amos>). The expression of TLR4 was evaluated by a semiquantitative RT-PCR. Technical details to the individual methods are provided in the Methods section of each particular paper.

Ethical note

All research included in this thesis was approved by either the Ethical committee of the Faculty of Science, Charles University in Prague or the Ethical committee of the Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, v.v.i.



Scarlet rosefinch study site near Želnavá village (photo M. Vinkler)

SUMMARY OF PAPERS

In the papers included in this thesis my co-workers and I investigated the health-signalling function of the carotenoid-based ornamental colouration in the Scarlet rosefinch. First, we revised the current literature and elaborated the theoretical background to our research in evolution of ornamentation [Papers I and II] and immunity [Paper IX]. Second, we examined the methodological tools used for the research in a model species of Zebra finch [Paper III]. Third, in the Scarlet rosefinch we confirmed the role of the carotenoid-based ornamental colouration in sexual selection [Paper IV] and examined the health state of the birds in our study population based on the haematological traits [Paper V]. Finally, we investigated the relationship between a chosen marker of inflammatory responsiveness and ornamentation [Paper X] and between the mate-choice and variability in immunity-related genes of the MHC complex [Paper VI]. We also proposed further research in the immunity-related genes and started analysis of a chosen PRR in a model species of Zebra finch [Papers VII and VIII].

Paper I:

Carotenoids and health signalling in animals

Vinkler M., Svobodová J., Maršík P. & Albrecht T. (scheduled to be published in 2011)

Published in: Yamaguchi M. (ed) *Carotenoids: Properties, Effects and Diseases*, Nova Science Publishers, Hauppauge, NY, USA; ISBN: 978-1-61209-713-8

Carotenoids represent one of the most common substances colouring the nature. We can find these in microbes as well as in plant and animal bodies where they have except for pigmentation many other important physiological functions. In animals carotenoid-based colouration is a common feature of many ornamental traits. In this review book-chapter we summarise the currently available theories and hypotheses concerning the possible role, signalling function and evolution of the carotenoid-based ornamentation. Special attention is paid to the signalling of the health and anti-parasite resistance based on these traits and the physiological processes preserving the honesty of this type of signalling, i.e. the essential preconditions for the process of the Parasite-mediated sexual selection.

Paper II:

Carotenoid maintenance handicap and the physiology of carotenoid-based signalisation of health

Vinkler M. & Albrecht T. (2010)

Published in: *Naturwissenschaften* 97:19-28; DOI: 10.1007/s00114-009-0595-9

Although many hypotheses concerning the carotenoid-based ornamental colouration assume health signalisation based on these traits, the underlying physiological mechanism remains unresolved. In this article we proposed a novel hypothesis which assumes that under certain conditions the maintenance of high levels of carotenoids is harmful to the organism. If antioxidant reserves are low, carotenoids may be easily attacked by reactive oxygen species resulting in their cleavage into toxic aldehydes. Therefore maintenance of high levels of carotenoids represents a handicap which can be afforded only by high quality individuals. We suggest that the physiological mechanism involves testosterone effect on antioxidant reserves and carotenoid levels. No specific original research has been performed to test this 'Carotenoid maintenance handicap' hypothesis. However, much of the evidence presently available and inconsistent with other models seems to support its predictions.

Paper III:

Functional analysis of the skin-swelling response to phytohaemagglutinin

Vinkler M., Bainová H. & Albrecht T. (2010)

Published in: *Functional Ecology* 24: 1081-1086; DOI: 10.1111/j.1365-2435.2010.01711.x

The phytohaemagglutinin (PHA) skin-swelling test is in immunoeology and ecotoxicology a widely used method to estimate the cell-mediated immunity and often presumed to reveal ‘T-cell mediated immunocompetence’. To investigate the immunological mechanism triggering this response we have compared in Zebra finch the immune responses to two PHA isolectins (PHA-L and PHA-E) differing in their biological properties and one control protein (bovine serum albumin). This is the first study to show the importance of erythroagglutination in the development of an inflammatory response to PHA-P (a commonly used mixture of the PHA-L and PHA-E isolectins). Our results indicate that the skin-swelling test using PHA-P reliably mirrors the individual general pro-inflammatory potential. The immunological background of the test is, however, highly complex and the test results cannot be interpreted as measurements of the adaptive immunity or T-cell activity. This interpretational change importantly alters our view on the test results regarding the costs of the response or the evolutionary immunological adaptations.

Paper IV:

Extra-pair fertilizations contribute to selection on secondary male ornamentation in a socially monogamous passerine

Albrecht T., Vinkler M., Schnitzer J., Poláková R., Munclinger P. & Bryja J. (2009)

Published in: *Journal of Evolutionary Biology* 22 (10): 2020-2030; DOI: 10.1111/j.1420-9101.2009.01815.x

In this study we test the hypothesis that extra-pair fertilizations (EPFs) contribute to the evolution or maintenance of male feather ornamentation in a sexually dichromatic passerine, the Scarlet rosefinch. We show that the colouration of ornamental breast feathers is a good predictor of basic sources of variation in male annual reproductive output in rosefinches and that the annual realized reproductive success of males is positively associated with measures of ornamental colouration only when gains and losses because of EPFs are considered. The results indicate that EPFs in rosefinches may rely on absolute (‘good genes’) rather than self-referential (‘genetic complementarity’) criteria of mate choice. Our study corroborates the potentially important role of EPFs in the evolution and/or maintenance of elaborate male ornaments in socially monogamous taxa.

Paper V:

Haematological health assessment in a passerine with extremely high proportion of basophils in peripheral blood

Vinkler M., Schnitzer J., Munclinger P., Votýpka J. & Albrecht T. (2010)

Published in: *Journal of Ornithology* 151 (4): 841–849; DOI: 10.1007/s10336-010-0521-0

Haematological traits may be important clues to the health and condition examination in free-living animals. In this article we show in the Scarlet rosefinch that haematological parameters other than the widely used heterophil/lymphocyte ratio may be important to investigate in birds. We give the full description of seven basic haematological traits. Most remarkably, we revealed that this species has an extraordinarily high proportion of basophils among the peripheral blood leukocytes (on average about 42 and 56%, respectively). Although the high basophil count is a general trait even in healthy individuals

of this species, the proportion of these cells is condition-dependent and is further increased by *Haemoproteus* infection. Our results also suggest that the immature erythrocyte count in the peripheral blood is a good predictor of the nestlings' growth rate and perhaps of the individual general metabolic rate. The rosefinch haematology differs strikingly from other avian species with known values of basic haematological parameters.

Paper VI:

Occurrence of extra-pair paternity is connected to social male's MHC-variability in the scarlet rosefinch *Carpodacus erythrinus*

Promerová M., Vinkler M., Bryja J., Poláková R., Schnitzer J., Munclinger P. & Albrecht T. (2011)

Published in: *Journal of Avian Biology* 42: 5-10; DOI: 10.1111/j.1600-048X.2010.05221.x

The MHC genes represent an essential component of the vertebrate immune system and their role in mate choice has been the subject of a particular scientific interest in the past decades. However, results of studies dealing with this topic in different species are equivocal and mechanisms conducting MHC-based mate choice are still puzzling. We studied the impact of MHC class I variability on within- and extra-pair fertilisation success in a socially monogamous passerine bird with considerable rates of extra-pair paternity, the Scarlet rosefinch. We found support for the 'good-genes-as-heterozygosity model', as social males of higher MHC-heterozygosity were cheated by females less frequently than the less MHC-heterozygous males. However, cuckolding males were not more MHC-heterozygous than the cheated social males, nor were extra-pair young more MHC-heterozygous than within-pair young. We did not find any evidence for mating preferences according to the 'complementarity' model.

Paper VII:

The question waiting to be asked: Innate immunity receptors in the perspective of zoological research

Vinkler M. & Albrecht T. (2009)

Published in: *Folia Zoologica* 58 (Suppl. 1): 15–28

This review paper suggests the importance of evolutionary and ecological research of the structure and polymorphism of innate immunity receptors in free-living animals. Giving the example of Toll-like receptors (TLRs) and their polymorphism in humans and domestic animals we point out the importance of variability in these receptors for resistance to various diseases. So far we have almost no information about the polymorphism of the genes for these or any other Pattern-recognition receptors in free-living animals. Thus also the evolutionary and ecological aspects of the polymorphism still wait to be studied.

Paper VIII:

Identification of the first Toll-like receptor gene in passerine birds: TLR4 orthologue in Zebra finch (*Taeniopygia guttata*)

Vinkler M., Bryjová A., Albrecht T. & Bryja J. (2009)

Published in: *Tissue Antigens* 74: 32-41; DOI: 10.1111/j.1399-0039.2009.01273.x

Toll-like receptors (TLRs) are the basic components of the vertebrate pathogen recognition system. Despite their uniform general structure, remarkable variability in domain composition can be found in individual TLRs among species. Knowledge of interspecific differences is of a particular importance to our understanding of the selective pressures forcing the TLR evolution. Currently, most TLRs are

characterized only in a limited number of model species, including domestic chicken as a universal avian model. Here, we describe structure and expression pattern of TLR4 in Zebra finch, a widely used passerine model species. The *tgTlr4* gene consists of three exons (204, 167 and 3033–3043 bp) that are transcribed into messenger RNA. Predicted protein is composed of 842 amino acids. The gene is highly expressed in the bone marrow and in the spleen. In mammals TLR4 protein is known to bind lipopolysaccharides originating from bacterial cell walls. Here we have shown for the first time in birds that expression of tgTLR4 in peritoneal macrophages is enhanced by the *Escherichia coli* lipopolysaccharide treatment.

Paper IX:

Handling ‘immunocompetence’ in ornithological studies: Do we operate with confused terms?

Vinkler M. & Albrecht T.

Submitted to: *Journal of Avian Biology*, 16th April 2011

‘Immunocompetence’ is a pivotal term in avian immunoecology used to refer to the ability of an individual to overcome potential parasite infections. However, as multiple ecological definitions of this term are presently used and all of them are liberal in immunological terms much of the potential intellectual interchange between ornithology and immunology is prevented. This decelerates the development of immunoecology as a scientific discipline. Although avian ecologists often aspire to obtain a single variable for immunity in their research, due to the enormous diversity of parasites possessing the ability to infect the host, there is no single value for anti-parasite resistance, i.e. no overall ‘immunocompetence’ *per se* exists. We propose to use the term ‘immunocompetence’ (defined as the ability to produce anti-parasite or anti-antigen immune response) only as a 0/1 character. The magnitude of a particular immune response (i.e. a continuous quantitative trait) should be referred to as ‘immune responsiveness’. Most importantly, the terms should always be used only with respect to a certain parasite taxon or antigen studied as otherwise they lose their explanatory value.

Paper X:

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

Vinkler M., Schnitzer J., Munclinger P. & Albrecht T.

Submitted to: *Animal Behaviour*, 23rd May 2011

Despite reasonable effort devoted to investigation of 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. In this study we therefore examined the linkage between several condition-dependent traits including red plumage ornamental colouration and the PHA-induced immune responsiveness in Scarlet rosefinch males. The histological analysis has revealed that the swelling response is dependent on basophil activity. We have also found relationship between the PHA-response magnitude and individual size, carotenoid-based ornamental colouration and a mean growth bar width. The positive linkage between the individual size or mean growth bar width and the PHA responsiveness suggests association of the magnitude of the response to individual metabolic rate. However, as the magnitude of the response was also negatively related to ornament saturation and positively to ornament brightness, our data indicate stronger responsiveness in inferior males.

GENERAL DISCUSSION

In the work included in this thesis my collaborators and I contributed to multidisciplinary investigation of the associations between immunity-related genes, immune function, carotenoid-based ornament expression and Parasite-mediated sexual selection in birds. Part of the work is purely theoretical while several particular field-and-laboratory tasks were solved in a model species of the Scarlet rosefinch with additional research being preformed also in the Zebra finch. Although reasonably heterogeneous by its composition the content of this thesis forms an integrated unit influencing several vital aspects of the problem in question.

The carotenoid-based ornamentation is used in many bird species as a clue for sexual selection (Hill 2002). Often the mate choice realised on the basis of these traits is primary, i.e. involving the formation of a social pair bond. However, in some avian species social pairs are formed irrespective to the mate quality. This may be, e.g., due to too hasty pairing enforced by a short breeding season (Spottiswoode & Møller 2004). This is also the case in the Scarlet rosefinch, a long-distant migratory species (Albrecht et al. 2007) in which the male quality does not affect its apparent reproductive success assessed based on its known social mating (Björklund 1990). However, we have shown that the male ornamental colouration has an important role in a cryptic extra-pair mating and EPFs [Paper IV of this thesis]. As more brightly ornamented males were more successful in obtaining extra-pair paternity and less susceptible to losing their within-pair paternity, male ornamentation importantly affected the realized annual reproductive success of the individual males. Nevertheless, what the message signalled by the male ornamental colouration is and why it is so important for the female mate choice remained unclear.

Many pieces of recent evidence to the signalling function of the carotenoid-pigmented ornaments suggest their role as health indicators [see Paper I], consistently with the predictions given by the Parasite-mediated sexual selection hypothesis (Hamilton & Zuk 1982). In the House finch, a species closely related to the Scarlet rosefinch the redness of the male ornamentation indicates its condition (Hill & Montgomerie 1994) and health (Hill et al. 2004). It has been also shown that in this species the more ornamented males are more resistant to some specific pathogen infections (Hill & Farmer 2005). Indeed, the results in the Scarlet rosefinch included in this thesis [Paper X] suggest that the male health may be signalled by its ornamental plumage colouration. However, to reach this conclusion we first need to clarify several partial aspects of the problem. First of all, we shall propose some mechanism capable of linking the feather colouration with the health state.

The mechanism enabling the individual health signalling through the carotenoid-based ornamental traits has received much attention in evolutionary ecology [see Paper I]. Most theories follow the predictions of the *Carotenoid-limitation hypothesis* suggesting that carotenoids are scarce in the nature and only individuals that are able to obtain and retain sufficient amounts of these pigments may develop the brightly coloured ornamentation (Lozano 1994; Perez-Rodriguez 2009). What differentiates the individual hypotheses (that are mostly not mutually exclusive) is the idea of the origin of the limitation in carotenoid intake or utilisation. The most original *Foraging ability hypothesis* (Endler 1980) proposed that the carotenoid-based colouration might signalise the individual abilities to obtain food resources of a sufficient quality or quantity. This hypothesis was later extended by the idea of a trade-off mechanism between carotenoid physiological function and deposition into the ornamental trait, the *Carotenoid trade-off hypothesis*. With respect to health signalling Lozano (1994) suggested that ornamentation might

compete with the immune system for the available sources of carotenoids and von Schantz et al (1999) proposed a similar mechanism for the antioxidative system. As carotenoid molecules are irreversibly destroyed in the process of antioxidative protection (Vershinin 1999) individual's antioxidative capacity would be linked to carotenoid-based colouration because carotenoids bleached by free-radicals cannot be deposited into ornamental traits any more. This concept of a trade-off between carotenoid utilization in free-radical quenching and their deposition into ornamental tissues is currently known as the *Antioxidant role hypothesis*. Later, this model was improved by addition of the hormonal regulation factor, forming the *Oxidation handicap hypothesis* (Alonso-Alvarez et al. 2007). Besides its immunomodulative and ornament-enhancing potential testosterone signalling also influences the oxidative balance of an organism by decreasing the resistance to oxidative stress. Finally, Peters (2007) proposed a model integrating the previous hypotheses on the physiological interactions behind the carotenoid-based signalling. In her view testosterone increases the susceptibility to oxidative stress on one hand (decreasing also the immune capacity of an organism), while on the other hand elevated testosterone concentrations make carotenoids available for the ornament expression. The carotenoids acting as antioxidants might therefore compensate for the immunosuppressive effect of testosterone. In other words, this model suggests that the immune system competes with ornaments for the available pool of immunostimulating carotenoids and hence the ornament elaboration is expected to be determined by the balance between the positive effect of testosterone on the bioavailability of carotenoids, and the negative effect of testosterone on the oxidative state and immunity. Contradicting this emerging paradigm, several authors stressed that carotenoids may not represent an important component in the antioxidant machinery in birds (Isaksson & Andersson 2008; Costantini & Møller 2008). Their reports fit the alternative view on the problem proposed by the '*Red herring hypothesis*' by Hartley & Kennedy (2004) which is assuming that colourful ornaments may not signal the carotenoid presence but rather sufficient levels of other, colourless antioxidants protecting carotenoids from the cleavage. The detailed description and supporting evidence to these individual hypotheses are given in the Paper I of this thesis. In our own work we carefully revised the presumptions of all the relevant hypotheses and proposed the principle of optimisation rather than maximisation of the carotenoid intake [the *Carotenoid maintenance handicap hypothesis*; summarised in the Paper II]. We followed the idea given by Olson & Owens (1998) suggesting that carotenoids may be risky. However, contrary to these authors and based on the most recent reports we assume the harmfulness of the carotenoid cleavage products. In our view the activity of carotenoids depends highly on the total antioxidative balance of the particular tissue. If antioxidant reserves are low, carotenoids may be easily attacked by reactive oxygen species resulting in their cleavage into toxic aldehydes. Therefore maintenance of high levels of carotenoids represents a handicap which can be affordable only to high quality individuals. This mechanism may involve a "double-edged-sword" regulation by testosterone. Although specific research is needed to test all the predictions of this hypothesis much of the evidence presently available fits its presumptions and it may serve as a valuable background theory for interpretation of the following results concerning the signalling of the health state based on colourful carotenoid-pigmented ornaments.

Health and disease are apparently only approximative categories describing opposite poles of a continual spectrum of physiological states. This enables us to think in the field of animal ecology in terms of 'more' or 'less' healthy, 'more' or 'less' sick. Any disease somehow lowers individual condition and thus reduces the resultant fitness (Clayton & Moore 1997). It is, nevertheless, a specific feature of a certain host and a certain parasite to what extent this is realised. The individual anti-parasite resistance is dependent on

both environmental and genetic factors [as discussed in the Paper IX of this thesis]. It is expressed in terms of the individual immunity which is specific to each individual parasite. This is also the reason why we proposed not to use terms like ‘immunocompetence’ unless the parasite or antigen in question is specified [see Paper IX]. To obtain a broader view, multiple tests are needed (Pedersen & Babayan 2011). However, we may choose to study in a closer detail only a subset of immunological features that may be in the best linkage to the issue of our study. In this thesis I decided to focus on the inflammatory immune response as this may be in the closest relationship to the redox balance of the tissue expressing the carotenoid-based ornamental trait [see Paper II]. The inflammation is a complex process arising from the activity of many different cell types (Campbell & Ellis 2007). By searching the literature (Goto et al. 1978; McCorkle et al. 1980; Martin et al. 2006) we have found that the commonly used PHA skin-swelling test is a method that might be useful to reveal the general pro-inflammatory potential of an individual. However, given the mitogenic activity of the PHA the method was often interpreted as a measurement of the T-cell activity or even T-cell proliferation. To test the relative importance of the mitogenic effect of PHA on induction of the inflammation we performed an experiment in Zebra finches with isolated PHA isolectins: PHA-L (mitogenic) and PHA-E (haemagglutinating). Contrary to the previously published conclusions by Tella et al. (2008) this experiment showed that the response is even greater when induced by the non-mitogenic PHA-E isolectin, thus revealing that the test measures non-specific inflammation [Paper III, please see also the discussion of the results by Tella et al. 2008 in this paper]. As stressed by Graham et al. (2011) the higher magnitude of any immune response does not necessarily mean better anti-parasite protection and maximisation of the host fitness. This is also true for the inflammation in which the previous health state has an important influence on the regulation of the response. In Scarlet rosefinch we performed a complex haematological screening to evaluate the health state of the birds in our study population. This study revealed an interesting aspect of the rosefinch immunology – practically all examined individuals had relatively high levels of basophils in their peripheral blood [Paper V]. Basophils are important cells of the avian immune system involved often in inflammatory processes (Maxwell & Robertson 1995) and the response to PHA is sometimes called as an cutaneous basophil hypersensitivity (Koutsos & Klasing 2008), given the large infiltration of basophiles into the skin swelling (Goto et al. 1978; McCorkle et al. 1980; Martin et al. 2006). In the scarlet rosefinch males basophils formed a predominant leukocyte type infiltrating the cutaneous inflammation induced by PHA application [Paper X]. In fact, the swelling response seemed to be mediated by basophils. Earlier we have shown the association between peripheral blood basophils and blood borne parasite diseases [Paper V]. Hence, our results indicate that the response is stronger in the inferior males while in the superior ones the intensity of the inflammation is lower. The results of our study show that in the Scarlet rosefinch the highly ornamented males responded significantly less by the cutaneous swelling triggered by the PHA treatment than the less ornamented ones. This is consistent with the assumptions of our hypothesis that the highly ornamented males are in better health, probably in better antioxidative balance and that the carotenoid-based ornamentation may provide an important signal for females with respect to their selection for the improved anti-parasite resistance in offspring.

Although some light has been shed on several aspects of the problem in question there are still many gaps remaining in our comprehension of the whole. One of these gaps lies undoubtedly in the genetic traits that differentiate the individuals with respect to their immunity and ornamentation and that are selected in the process of the Parasite-mediated sexual selection. In our MHC work my colleagues and I have shown that the social males being highly MHC-heterozygous were less frequently cheated by

females than the less MHC-heterozygous males [Paper VI]. Nevertheless, the cuckolding males were not more MHC-heterozygous than the cheated social males, nor were extra-pair young more MHC-heterozygous than the within-pair offspring. Thus, we did find some support to the ‘good-genes-as-heterozygosity model’ but the matter is far from being resolved as at least based on MHC class I variability we found no evidence for females gaining any indirect advantage from mating outside the pair-bonds. Our results contrast with those reported earlier by Richardson et al. (2005) in Seychelles warblers (*Acrocephalus sechellensis*) or Bonneaud et al. (2006) and recently Griggio et al. (2011) in House sparrows (*Passer domesticus*). It is also possible that the genetic traits we focused on were wrong or insufficient. As highlighted by Acevedo-Whitehouse & Cunningham (2006), MHC may not be enough to understand the wildlife immunogenetics. To contribute to filling this gap in our knowledge we proposed a research focused on the genes encoding molecules involved in innate immunity – most conveniently the Pattern-recognition receptors [Paper VII]. These molecules have been shown even in birds to be highly variable (Downing et al. 2010; Alcaide & Edwards 2011) and with direct associations of the polymorphism to the disease resistance (Leveque et al. 2003). We started mapping these genes in Zebra finch as a representative of passerine birds and showed in the TLR4 of this species that the gene is reasonably polymorphic [Paper VIII]. However, the impact of this polymorphism in innate immunity genes in birds still remains unresolved and I hope this subject will gain more attention in the future.

GENERAL CONCLUSION

Ecological and evolutionary immunology is a rapidly developing branch of the modern interdisciplinary biological research (Ardia & Schat 2008). In the work included in this thesis my co-workers and I tried to improve the current knowledge to one of its most vivid issues: the topic of the involvement of sexual selection in the evolution of anti-parasite resistance. In the model species of Scarlet rosefinch we have shown that the male carotenoid-based ornamental colouration is an important trait for the cryptic extra-pair mate choice in this species. Male success in the extra-pair mating forms a significant part of variation in the male annual reproductive success and thus it has important fitness consequences. The carotenoid-pigmented traits serve in many species as signals of individual health. Based on the known immunological background of the PHA skin-swelling test and the haematological state of the birds in our study population we have shown also in the Scarlet rosefinch that the extensively ornamented males are healthier than the less ornamented ones. To explain the principle of the maintenance of honesty in the male carotenoid-based display we proposed the Carotenoid maintenance handicap hypothesis which seems to fit most of the evidence currently available. This hypothesis highlights sustainability of optimisation rather than maximisation of the carotenoid quantities in the male organism due to the potential detrimental effect of the carotenoid cleavage. In the Scarlet rosefinch we investigated also the relationship between the mate choice and variability in immunity-related genes of the MHC complex. We showed that social males of high MHC-heterozygosity were cheated by females less frequently than the less MHC-heterozygous males. Hence, our findings supported the 'good-genes-as-heterozygosity' hypothesis although still much ambiguity remains in the results. Finally, we proposed and started further research in the immunity-related genes focused specifically on innate immunity.

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LIST OF ABBREVIATIONS

DNA	–	Deoxyribonucleic acid
EPF	–	Extra-pair fertilization
MHC	–	Major Histocompatibility Complex
PHA	–	Phytohaemagglutinin
PCR	–	Polymerase chain reaction
PRR	–	Pattern recognition receptor
RACE	–	Rapid Amplification of cDNA Ends reaction
RNA	–	Ribonucleic acid
SSCP	–	Single-strand conformation polymorphism analysis
TCR	–	T-cell receptor
TLR	–	Toll-like receptor

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- I. **Vinkler M.**, Svobodová J., Maršík P. & Albrecht T.: Carotenoids and health signalling in animals. In: Yamaguchi M. (ed) Carotenoids: Properties, Effects and Diseases, Nova Science Publishers, Hauppauge, NY, USA; ISBN: 978-1-61209-713-8; scheduled to be published in 2011
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- II. **Vinkler M.** & Albrecht T. (2010): Carotenoid maintenance handicap and the physiology of carotenoid-based signalisation of health. *Naturwissenschaften* 97:19-28; DOI: 10.1007/s00114-009-0595-9
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- IX. **Vinkler M.** & Albrecht T.: Handling 'immunocompetence' in ornithological studies: Do we operate with confused terms? (submitted to *Journal of Avian Biology* in April 2011).
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- X. **Vinkler M.**, Schnitzer J., Munclinger P. & Albrecht T.: Phytohaemagglutinin skin-swelling test in scarlet rosefinch males: low-quality birds respond stronger (submitted to *Animal Behaviour* in May 2011).
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Paper I

Chapter 8

CAROTENOIDS AND HEALTH SIGNALLING IN ANIMALS

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ABSTRACT

Carotenoids belong to biochemicals essential in the animal diet. This is due to their indispensability in several life-preserving physiological processes. In many species, however, carotenoids serve also as the main pigments colouring various ornamental structures engaged in mate choice. Although intensively studied by biologists, the evolutionary causes for sexual selection mediated by carotenoid-based ornaments remain unclear. Much of the evidence currently available indicates that carotenoids may signalise individual's health status. But how is this achieved? Why a substantial level of variability still exists among individuals despite the long-term natural selection posed on these traits? Indeed, one of the most puzzling questions of modern evolutionary ecology concerns the physiological mechanisms preserving the honesty of the signalling through carotenoid-pigmented traits. A number of different hypotheses has been proposed to explain this phenomenon. One of the most widely accepted hypotheses connects ornamental health signalling via carotenoids with their antioxidative function in the animal bodies. According to this view, carotenoids important for protection of the organism against oxidative damage are irreversibly destroyed in the antioxidative process and hence only healthy animals with sufficient carotenoid intake and low oxidative burden may afford elaborated ornamentation. However, most recent findings cast some doubts on this explanation. Carotenoids are not the only antioxidants available in the tissues and most probably they are not even the most important ones. Novel alternative hypotheses based on indirect signalling and the handicap principle appear to provide a possible key to resolve the riddle. If carotenoids deposited into ornamental tissues indicate the presence of other substances than carotenoids themselves or if carotenoid cleavage into toxic products causes additional harm to the animal health then honesty of the carotenoid-based signalling may be preserved. Given that carotenoids are commonly used as dietary supplements, research in this field is not only a matter of an academic importance but may provide valuable implications both for animal and human food production and pharmacy.

Keywords: Carotenoids, condition, diseases, health, ornamentation, oxidative stress, parasites, sexual signalling

INTRODUCTION

Since their discovery in 19th century, carotenoids have been known to function as micronutrients essential for human health. Much less widely known, however, is their property as one of the most important pigments colouring the nature that surrounds us. Carotenoids are precisely the substances that make bird feathers, beetle elytra and autumn leaves bright in colours from yellow to red (Vershinin 1999; Matile 2000; Bezzerides et al. 2007). Even human skin is partially coloured by carotenoids (Alaluf et al. 2002) although this may not be clearly visible at first glance. In many other vertebrate taxa (e.g. in various birds or fish), on the contrary, carotenoids may create conspicuous integument colouration and in the form of ornaments serve as colourful signals on prominent body parts. But what is the reason for the existence of these traits in animals? What is the information they encode? Is it valuable for the signal recipient? These and other questions have preoccupied the minds of many scientists ever since the formation of modern zoology and evolutionary biology. Researchers soon realized that a substantial level of variability often exists among individuals in the elaboration of these colourful traits (Andersson 1994). For instance in the House finch (*Carpodacus mexicanus*), a common American passerine, it is not uncommon to observe on the same feeding grounds males that are coloured brightly red alongside others shaded pale or yellowish (Hill 2002). Charles Darwin was first to recognise the true importance of the variability in ornamental signals for animal communication. In his opinion, an individual's reproductive success is simultaneously governed by both the ability to survive and to attract an optimal sexual partner (Darwin 1871). The latter phenomenon is called *sexual selection* and it took more than half a century before it became an intensively studied area of evolutionary biology. Despite this initial lack of attention, sexual selection nowadays attracts significant interest amongst researchers in this field. At present, most scientists accept that sexual selection has been the force which presumably promoted the appearance of many sex-specific phenotypic traits, some of which are highly remarkable. Although much effort has been devoted to investigating mechanisms by which sexual selection operates and a valuable theoretical framework has been elaborated, our understanding of this topic is still limited. The evidence presently available is unfortunately rather inconsistent in many of its details. This is also true for the mechanisms preserving the long-lasting interindividual variability in carotenoid-based colouration which is believed to reflect an individual's health (Badyaev & Hill 2000). In the following text we critically review current hypotheses and models to this topic aiming to highlight, with the evidence available, their strong points as well as weaknesses. To provide a coherent view we discuss all relevant aspects of the carotenoid-based signalling, focusing mainly on its evolution and physiology.

EVOLUTION OF ORNAMENTAL SIGNALLING OF INDIVIDUAL'S HEALTH

Sexual dichromatism, i.e. difference in colouration between males and females, is probably one of the most striking examples of sexual selection outcomes. In carotenoid-based colouration sexual dichromatism is particularly common with males often being the more ornamented sex. For simplicity, in the following text we will deal mostly with male ornamentation. Similar mechanisms, albeit usually of lower intensity, may, however, operate on the female sex as well (Amundsen et al. 1997; Amundsen & Forsgren 2001).

The question of how these ornamental sexual traits appear is thus far difficult to answer. In principle there are four main groups of models describing the evolution of ornamental traits (Jennions & Petrie 2000): (1) Fisher's 'run-away' model, (2) Viability indicator model, (3) Sensory exploitation model and (4) Antagonistic seduction model.

The *Sensory exploitation model* and the *Antagonistic seduction model* are built on the simple presumption that direct evolution of the female ornament preferences are driven by natural selection (Jennions & Petrie 2000). In view of these two models it was the natural selection pressure imposing on other aspects of animal ecology that made females concerned about a particular feature. This feature was later exploited by male ornamentation leading in females to non-adaptive preference of the signal-bearer as a mating partner (Ryan 1998). In other words, both hypotheses assume some sensory bias in females which governs their mate choice (Jennions & Petrie 2000). For instance, the female may be biased towards selection of a brighter red colour because it is easy for her eyes to detect. In that case, evolution of brighter red ornamental colouration in males can be predicted. The main difference between the Sensory exploitation model and the Antagonistic seduction model is that the latter considers evolution of intersexual conflict, predicting a counter-selection for female resistance to the trait preference followed by cyclic antagonistic co-evolution between male ornament exaggeration and female preference resistance (Holland & Rice 1998). Although currently less often tested, less widely accepted and less supported by experimental evidence than the other two models, the principle of the sensory bias may be particularly useful to explain the origin of the female attraction towards the male signals.

Proposed by R. A. Fisher in 1930 (Fisher 1930) the *Runaway model* was in fact the first rigorous hypothesis aiming to explain the evolution of ornamental traits. To describe the basic principle of the model, let us assume a population in which an allele appears determining a female preference for a particular male ornamental trait.

Whereby the allele for this preference has reached a significant frequency in the population, it is likely that this would be reflected by the appearance of an appropriate novel heritable ornamental trait in males. Males possessing this trait will be more capable of obtaining females with the corresponding preference, which will lead to assortative mating. In combination with sexual selection favouring attractive males this establishes a linkage disequilibrium between genes for the ornamentation and genes for the preference (Mead & Arnold 2004). As a result, the more females that prefer the trait in the current generation the more males will possess it in the next generation (as these are being sired by the ornamented males). In addition more female offspring will inherit the allele for the preference of the ornamented males from their parents. Rigorous choice of mating partners may be costly to females. In such a situation the cost of the choosiness arising from the preference allele must be compensated by the fitness benefits rising from the mate choice (see e.g. Pomiankowski & Iwasa 1998). If so, we expect a self-reinforcing mate-choice mode to occur. This mechanism may be responsible for a rapid spread of the alleles for the ornamental trait and for the preference in a population (Burt 1995). This principle is central to a model called the *Hypothesis of 'sexy sons'*, predicting fitness gains resulting from the selection for the ornamented males. Males expressing the ornament are 'sexy' to females wherefore it is advantageous for both males and females to produce offspring bearing this trait as it ensures relatively high individual fitness gains. This model is commonly assumed for quantitative traits that are influenced by multiple gene loci. In this case a correlation may appear between the strength of the preference and the elaboration of the trait leading to a runaway effect on the trait expression. The fact that females in every generation tend to choose as mates males with most elaborated ornaments promotes in males the expression of even more elaborated ornamental traits. The ornament evolves at ever-increasing speed under the force of ever-stronger preference (Mead & Arnold 2004). This process of ornament and preference exaggeration is not stopped until the extreme expression of ornamental trait is balanced by oppositely directed natural selection pressure. However, as a consequence of the extremely skewed mating success amongst males, a reduction in genetic variation among males may eventually occur leading to a decrease in variability in the ornamental trait (Burt 1995). If the alleles involved become fixed within the population there remains no further clue for the female mate choice and the system collapse. This problem is well known as a *Lek paradox* (see e.g. Pomiankowski 1990; Hamilton et al. 2006) and beside the Genic capture mechanism (Tomkins et al. 2004; Birkhead et al. 2006) it may also be resolved by adoption of the Viability indicator mechanism.

According to the Fisherian 'Sexy son' runaway model more ornamented males only possess higher fitness due to their higher attractiveness to mating females (Pomiankowski & Iwasa 1998). In other fitness components, such as health, the 'sexy' males are hypothesised to be equal to less ornamented ones. Thus the mate choice does not influence the viability of the offspring, although the male offspring is more attractive to females. In contrast, the *Viability indicator model* assumes that the trait serving as a criterion for the female mate choice honestly indicates male quality with respect to its viability, health and vigour (Andersson 1994). Such a choice for high-quality males may then influence the ability of the offspring (both male and female) to survive either directly or indirectly. More ornamented high-quality males may provide direct benefits, for instance in the form of better food provision or nest protection. The ornament may thus indicate a non-heritable component of parental quality. This is the central assumption of the *'Good parent' hypothesis* (Hoelzer 1989; for supportive evidence see e.g. Senar et al. 2002). According to the *Contagion indicator hypothesis* females and offspring may also directly benefit from lower risk of disease transmission if more ornamented males are healthier and thus less likely to spread illnesses (Able 1996). In contrast the *'Good genes' hypothesis* assumes that females choose their mates according to phenotypic traits that serve as honest signals of some desirable heritable trait (Neff & Pitcher 2005).

As mentioned above, the question of maintenance of long-lasting within-population variability in male secondary sexual traits, i.e. the Lek paradox is a central problem of the Runaway model. The Viability indicator model assumes that the presence of the intraspecific variation in well-established ornamental traits reflects the interindividual variability in trait-expression limitation. This is consistent with the Zahavi's (1975) *handicap principle* – to preserve honesty in signalling secondary sexual traits must be costly to produce or maintain. Without this limitation, whatever it is, all males would after an evolutionary short period express their ornaments in the maximal possible level and females would lose their clue for discrimination. Nevertheless, if there is some limiting factor influencing the individual condition in a differentiating manner (such as a resource acquisition ability, Hill 2002, or anti-parasite resistance, Hamilton & Zuk 1982) and the individual condition then, in turn, determines the ability to express the ornamentation, sufficient levels of variability among individuals may be maintained in ornamentation. Selection pressure is, thus, supposed to act on genes influencing the ornamentation but not directly encoding the pigment deposition pattern. Those males who cope better with the cause of limitation shall be able to produce better ornamentation and this ornamentation may serve to females as an honest signal of the male genetic quality. Hence the Viability indicator model might explain preservation of genetic variability despite continual selection of secondary sexual traits and resolve the Lek paradox.

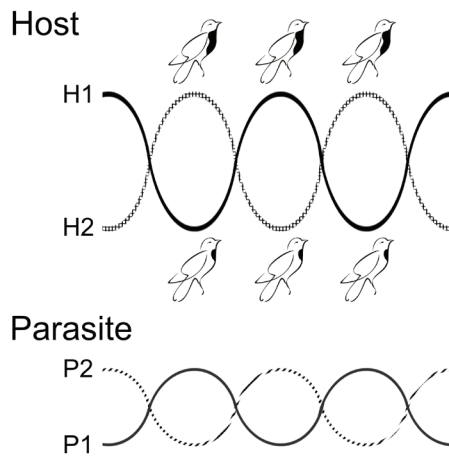


Figure 1. Parasite-mediated sexual selection: A schematic representation of cyclical changes in host (H1, H2) and parasite (P1, P2) allele frequencies. Parasites possessing alleles compatible to host genotype (P1 to H1 and P2 to H2) are more efficient in infecting the host, thus lowering its condition and ornamentation. Level of ornament expression therefore reflects the infection status and contemporary protective advantage of the host genotype. Allele frequencies in host and its parasite change in cycles. This is based on contemporary selection advantage of host genotype increasing the allele frequency and subsequent spread of parasite genotype co-adapted to this host genotype and decreasing the allele frequency (designed according to the model given by Hamilton & Zuk 1982).

Among the viability indicator models the emergence of the *Parasite-mediated sexual selection hypothesis* proposed by Hamilton & Zuk (1982) was particularly influential. Hamilton & Zuk (1982) suggested that individual alleles of anti-parasite-resistance genes vary in their protective effect over time in a frequency-dependent manner. This is because parasites and their hosts evolve in co-adaptational cycles in which the relative advantage of individual host alleles shifts with the actual prevalence of individual parasite genotypes. These cycles create a continual source of variation in host genotypes. Ornamentation is assumed to be condition-dependent and the condition itself is influenced by health status. Hence, the variation in the ornamental trait may be maintained by the cyclical changes in the momentary protective advantage of the competing host genotypes (see Figure 1). Females choosing a partner according to the extent of ornament elaboration choose for their offspring in every phase of the cycle different alleles providing them with contemporary anti-parasite resistance. In this process all female benefits are indirect, based solely on the quality of the genetic information the male passes to its young. Therefore, this is a plausible hypothesis to explain the benefits to the females from extra-pair copulations where females consider in their mate choice the male ornament elaboration (see e.g. Jennions & Petrie 2000).

The Parasite-mediated sexual selection model is rather simplistic. We are now aware that the complicated architecture of the vertebrate immune system makes the condition dependent on the effect of many different genes (see e.g. Birkhead et al. 2006). Still, there is a reasonable amount of evidence to support the Parasite-mediated 'good genes' model. For example the relationships between ornamentation and parasite load (Thompson et al. 1997; Brawner et al. 2000; Figuerola et al. 2003; Hill et al. 2004; Fenoglio et al. 2004; Hill & Farmer 2005) and health-related traits (Dufva & Allander 1995; Figuerola et al. 1999; Fenoglio et al. 2002; Fenoglio et al. 2004) were found in various species. Also the heritable nature of the variability in disease resistance is becoming increasingly apparent (see e.g. Sommer 2005; Vinkler & Albrecht 2009). We should realise that the effect of polymorphism in individual immunity genes may have only modest protective effect on health. The variance in anti-parasite resistance may not result directly in differential mortality but in the variable state of individual's condition. As shown by many studies, mounting an immune response may be costly and even these costs may result in lowered ornamentation (Schmid-Hempel 2003; Faivre et al. 2003a; Alonso-Alvarez et al. 2004; Peters et al. 2004a). Therefore genotypes enabling quicker and easier defeat of parasites are advantageous for ornament elaboration and increased reproductive success.

The evolutionary processes described by most of the models mentioned above may take place simultaneously or successively in the evolution of ornaments. Let us assume the appearance of an allele driving female preference to male bright red colouration in a bird population. Such an allele may first appear as a reflection of some former predisposition and a sensory bias. As described by the Sensory exploitation model it may be a successful male strategy under these circumstances to take the advantage of the situation and express adequate ornamentation. The tendency of females to copy the mate choice of others (Pomiankowski 1990) may then contribute to higher attractiveness of ornamented males. This mechanism could further promote the spreading of the allele for the preference and the allele for the ornamentation throughout the population. When the frequency of the preference allele is substantially high, the mechanism of the Fisherian runaway process starts to be employed. It is now convenient for females to mate with ornamented males as this ensures that their own sons will be attractive to other females and thus their fitness will be high. The genetic correlation between the preference gene and ornamental genes will then lead to rapid exaggeration

of the ornamental trait and reinforce the preference to the most ornamented males. The ornament development is later stopped by the opposing pressures of natural selection. However, before the fixation of the alleles enabling maximal expression of the ornamental trait can occur, a transition from the Fisherian runaway processes to the Viability indicator mechanism (such as the ‘Good genes’ process) would take place (Balmford & Read 1991). For instance, highly expressed red colouration is costly due to insufficient access to carotenoids in the environment and these become limiting. Only some of the males, depending on their condition and health, are capable of expressing the ornament in its maximal level and these are selected by females. The ornamental trait gains a new function as a signal encoding the information on the male health which may be determined by its genotype. Hence, the ‘Good genes’ mechanism as expected by the Parasite-mediated sexual selection hypothesis may be preceded and reinforced by the runaway processes (Mead & Arnold 2004) and also by the effect of parasite contagion as proposed by the Contagion indicator hypothesis (Able 1996). Indeed, a continuum is sometimes expected to be formed by these two processes (Kokko et al. 2002; Griffith et al. 2002). Moreover, during species evolution the indicatory function of different traits may diversify and several distinct morphological or colourful ornamental traits may gain independent information values; some referring to the individual dominance, some to the condition and health (Gonzalez et al. 1999; Badyaev & Young 2004; Fenoglio et al. 2004).

Many different hypotheses were proposed to explain the direct mechanism linking the ornament expression to anti-parasite resistance. Some of them are based on a physiological handicap (Folstad & Karter 1992; Wedekind & Folstad 1994; Hartley & Kennedy 2004; Vinkler & Albrecht 2010) while others expect some direct trade-off in resource distribution (Poiani et al. 2000; Hill 2000; Peters 2007). These hypotheses are discussed in detail and with respect to carotenoid-based colouration in the following subchapters.

CAROTENOID-BASED PIGMENTATION OF ORNAMENTAL TRAITS

In most sexually reproducing animals, sexual selection is stronger in the male sex (Andersson 1994). This is because variance in reproductive success is typically higher in the sex investing less into reproduction, which in most cases, is the males (Shuster & Wade 2003). This led Darwin (1871) to consider sexual selection as the basic mechanism responsible for the evolution of sexual dimorphism, a state in which males differ from females in phenotypic traits. There is no doubt about the evolutionary causes of sexual dimorphism in primary sexual traits, such as genitalia morphology. The situation is more complicated when it comes to secondary traits such as ornamental colouration. In this subchapter we focus on carotenoid-based colouration, mainly in males, since, as noted above, this is the sex in which sexual selection is typically more intense.

Yellow-red carotenoids represent just one type of pigment used by animals to paint their bodies. Melanins form another common source of colouration, causing reddish-brown and black colouration (Ducrest et al. 2008). The third widespread type of colouration is the structural-based, often UV-reflecting colouration detected in some invertebrates, fish, reptiles, birds and even mammals (e.g. Rhode et al. 2001; Siebeck & Marshall 2001; Hausmann et al. 2003; Hunt et al. 2009; Guz et al. 2010). Despite originally brightly red, in combination with these other pigments (melanins and various keratin derived structures), carotenoids may gain even green and ultraviolet colouration (Prum 2006).

With respect to carotenoid-based ornamentation, birds represent by far the most studied group of animals. Besides birds, carotenoid-based colouration occurs in various forms in many other taxa, including fish, reptiles, amphibians and some invertebrates (e.g. Rhode et al. 2001; Van Nieuwerburgh et al. 2004; Karino et al. 2010, see also below). Although carotenoid pigmentation may also have other functions (for example to provide protection against UV light, Rhode et al. 2001), its role in sexual selection is assumed in most cases. In this context xanthophylls are mainly utilised (Møller et al. 2000). Elaboration of the carotenoid-based ornamentation, i.e. its colour intensity, may substantially vary even within species with the level of ornament expression partially heritable (Hill 1991) and partially dependent on the environment (Brush & Power 1976). Carotenoid-based ornaments are assumed to signal male quality in terms of competitive abilities, foraging abilities or the quality of immune system and pathogen resistance (Hill 2002; Blount et al. 2003; Peters 2007; Pike et al. 2007c; Vinkler & Albrecht 2010).

In invertebrates, carotenoid based colouration may form an important component of aposematic signals. Here, carotenoid-based colouration may honestly signal individuals’ quality in terms of its non-edibility and concentration of poisonous chemicals in its body. It has been found that the poisonous larvae of a moth *Orgyia antiqua* are polymorphic in their colouration, with only some having conspicuous yellow hair tufts. An analysis of the colour of these hair tufts revealed a presence of lutein (Sandre et al. 2007). How the colour polymorphism is maintained in natural populations remains unclear and no associations between larvae quality (growth rates and mortality) and the presence of carotenoid-based colouration have been found under lab conditions (Sandre et al. 2007). On the other hand, in poisonous ladybird beetles, the extent of carotenoid based patches reflects alkaloid concentration (Bezzerides et al. 2007), supporting the idea of a link between individual quality and aposematic signals composed of carotenoids. Interestingly, carotenoid-based colouration of elytra in ladybird beetles (Britton et al. 1977) may also act as an ornament, with redder individuals being more attractive as mates (Hodek & Ceryngier 2000).

In fish, carotenoids occur in various tissues, including the retina, the skin and the integument (see e.g. Hudon et al. 2003; Reichard et al. 2009). Carotenoids (mostly 4-oxo-carotenoids, astaxanthin and cantaxanthin) are also deposited into the muscles of salmonid fish, being responsible for rubicund colouration of the flesh (Matthews et al. 2006). The role of carotenoid-based signals in sexual selection has mainly been studied in the Three-spined sticklebacks (*Gasterosteus aculeatus*) and Guppies (*Poecilia guttata*). In Guppies, carotenoids are responsible for orange integumental spots, predominantly composed of esters of tunaxanthin (Hudon et al. 2003). Concentration of carotenoids in the orange spots of male guppies is five to nine times higher than in rest of the integument (Hudon et al. 2003). Substantial evidence shows that the carotenoid-based ornamentation plays a role in sexual selection in this fish with more ornamented males being preferred by females over the less coloured ones (Houde 1987; Kodricbrown 1993; Godin & Dugatkin 1996). Male attractiveness seems to be a function of size of the carotenoid-based spots (Karino et al. 2010). Colourful males also produce faster and more viable sperm indicating that carotenoid-based colouration serves as a signal of competitive abilities of sperm cells in the process of fertilization (Locatello et al. 2006). Brightly coloured Guppies are, however, exposed to increased predation risk, indicating the existence of a survival cost arising from the highly elaborated carotenoid-based sexually selected trait in this fish (Godin & McDonough 2003). Yellow to red nuptial colouration of breeding Three-spined stickleback males is composed mostly of tunaxanthin/lutein and astaxanthin (Wedekind et al. 1998). Also in this species, redder males are more attractive to females (Milinski & Bakker 1990) and similar results are known from other fish species with a similar type of skin colouration such as the Fighting fish (*Betta splendens*) and Cichlid (*Pundamilia nyererei*; Maan et al. 2006; Clotfelter et al. 2007).

Much less is known about the role of carotenoid-based ornamentation in amphibians and reptiles, although many species in these groups possess this type of pigmentation (Cote et al. 2010; Steffen et al. 2010). In several species, carotenoid-based colour patches are bigger in males than in females, indicating a possible role of these ornaments in sexual selection (Davis & Grayson 2008). Ornamentation in lizards is often made of carotenoids (xanthophylls) in combination with pterins and melanins, i.e. pigments that can be synthesized in animal bodies (Macedonia et al. 2000; Steffen & McGraw 2007). In some gecko species, a high concentration of carotenoid pigments (possibly galloxanthin) has been detected in the retina and lens (Roll 2000).

Birds represent a group of vertebrates in which carotenoid-based ornamentation is both widespread and well studied. Carotenoids are responsible in birds for yellow-red colouration of feathers, skin, skin derivatives (podotheca and ramphotheca) and retina (Hill 2006). The total carotenoid content in the tissue correlates with hue and especially saturation of the ornamental structure (Saks et al. 2003a). As with other taxa, carotenoid-based ornaments in birds can be used as signals in various contexts (Møller et al. 2000). Basically, carotenoid-based ornaments are thought to play a role in mate choice and male-male contests and status signalling (Hill 1990; Hill 1991). Although most of the evidence available on the role of carotenoid-based colouration in birds is correlative, there are also some experimental studies showing a clear effect of carotenoid-based colouration on male attractiveness (Møller et al. 2000). In the House finch, the hue of feather ornamentation varies from yellow to red, as a function of the concentration of red 4-oxo-carotenoids (Inouye et al. 2001). In experimental mate choice tests, redder males were clearly preferred over yellow ones by females (Hill 1991). Experimental manipulation with diet and pathogen loads has shown that red plumage can only be acquired by high quality individuals (reviewed in Hill 2002). Whether this quality can be inherited by offspring remains unclear. However, in a correlative study, a link between the ornamentation of males and their male offspring has been found (Hill 1991). Not only feather ornaments, but also other skin derivatives and bare parts may signal individual qualities via carotenoids (Hill 2006). Feather ornaments may provide information only about individual quality at the time of moult, when carotenoids are deposited into new follicles (Hill 2002), whereas continuously growing skin derivatives, such as ramphotheca, mirror immediate changes in individual condition (Faivre et al. 2003a).

In mammals, the carotenoid-based colouration of ornamental traits is uncommon. Nevertheless, even here we can find indication for a mate-choice based on carotenoid pigmentation. For instance, human skin is known to be partially coloured by its carotenoid content (Alaluf et al. 2002). Recently it was found that the attractiveness of human faces is increased to some extent by more intense yellowish colouration connected with a healthy appearance of the skin, which indicates the influence of the carotenoid skin deposits (Stephen et al. 2009).

CHEMICAL CAUSES OF CAROTENOID-BASED COLOURATION

Due to their unique functions in plant and animal organisms, carotenoids are the most widely spread colouration substances in nature. Presently, about 700 naturally occurring carotenoids have been identified (Britton et al. 2004). In plants carotenoids are found in chloroplasts in green tissues or in specialized organelles termed chromoplasts in sink organs such as roots, fruits or flowers (Galpaz et al. 2006). As animal cells do not possess the ability to synthesise carotenoids, animals have to obtain carotenoids from their diet, i.e. from plants or other animals (Vershinin 1999).

In plants carotenoids are synthesized from activated isoprene isomeric forms: isopentenyl diphosphate and dimethylallyl diphosphate (Buchanan et al. 2000). The biosynthesis of these precursors can start via two pathways

differing in their intracellular localization as well as in the mechanism of synthesis (Lichtenthaler 1999). In the first pathway, the plastid-localized methylerythritol 4-phosphate route, carotenoid precursors are produced in an evolutionary more original fashion by a mechanism related to carotenoid synthesis in eubacteria (Eisenreich et al. 2001). Alternatively, carotenoid precursors may be formed by a mevalonic acid pathway. This route is more common and is situated mainly in the cytosol, although it participates in plastid-carotenoid biosynthesis as well (Farre et al. 2010). In the next step isoprene conjugates into a long chain (Buchanan et al. 2000). This is realized in plastids. The schematic visualisation of the biosynthesis is given in Figure 2. Possessing lipophilic features, final forms of carotenoids are located (both in plant and animal tissues) mostly in membranes or intracellular lipid bodies, often forming complexes with proteins or conjugates as fatty acid esters (Taiz & Zeiger 1998; Hornero-Mendez & Minguéz-Mosquera 2000; Grynbaum et al. 2005).

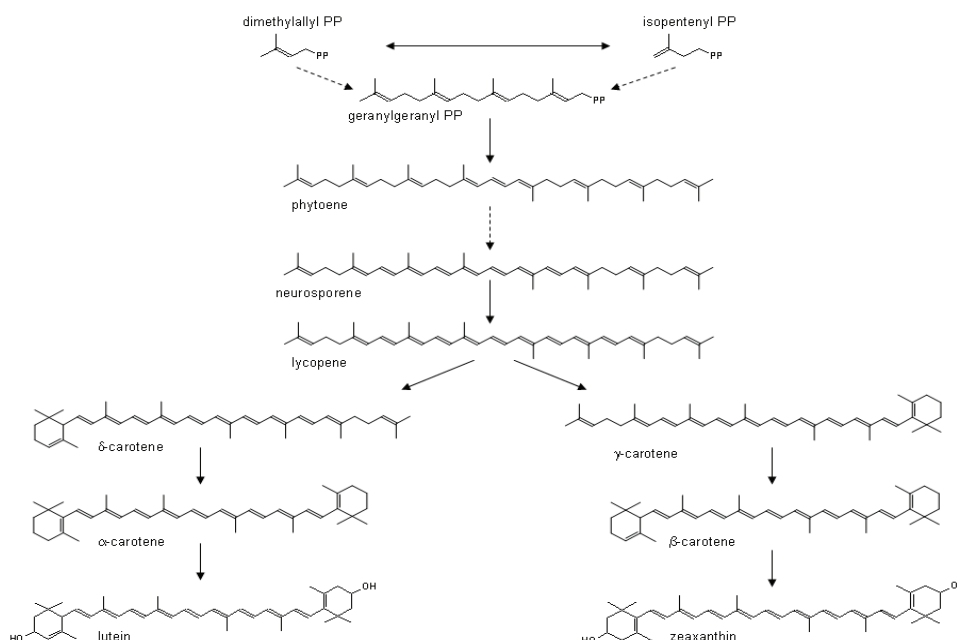


Figure 2. Simplified scheme of carotenoid biosynthesis in green plants (according to Burns et al. 2003).

From chemical point of view, carotenoids belong to tetraterpenes (Clayden et al. 2000). Their molecules consist of eight condensed isoprene units, usually with 40 carbons in total. The carotenoid molecule family is a very diverse group of substances, including linear structures (lycopene), structures with one or two end cycles or aromatic rings. Carotenoids are divided into two groups according to the presence of heteroatoms (oxygen) in the molecule: (1) *carotenes* are carbohydrates without any heteroatoms while (2) *xanthophylls* possess hydroxy-, keto-, epoxy- or aldehydic groups in their chains. Several carotenoids contain also acetylenic (triple bond) or allenic (two non-separated double bonds) groups in the aliphatic parts of their molecules (Britton et al. 2004).

The structural specificity providing carotenoids with their typical colouration and unique chemical features is the accumulation of conjugated double bonds in the aliphatic part of their molecules (Vershinin 1999). This conjugated system is called chromophore and is crucial for the spectral properties of the molecule. The structural arrangement of the chromophore containing delocalized π -electrons enables easy absorption of photons. The photon absorption promotes the delocalized π -electrons to a free anti-bonding orbital, leading to the higher-energy state of the molecule (Britton 1995). Electrons in chromophores are highly delocalized wherefore the energy for their transition is relatively low. Therefore, the energy of visible light is sufficient for molecular electronic transition. Most carotenoids absorb light maximally between 400-500 nm, which corresponds to blue, in extremes blue-violet and green-blue colours. The increasing chromophore length reduces excitation energy of bounding π -electrons, which consequently prolongs the absorbed wavelength (Cogdell et al. 1994). With an increasing number of double bonds involved in the chromophore, the remaining complementary colour, i.e. the resultant colour of the pigment, becomes near to red. The carotenoid colour is also highly dependent upon the functional group. For instance, the keto-groups on the end rings of canthaxanthin or the allene groups of neoxanthin shift the absorbed spectrum, either towards shorter (allene) or longer (conjugated ketone) wavelengths. Other functional groups such as hydroxy- group in lutein or zeaxanthin, on the other hand, have no or only a weak effect on light absorption (Britton et al. 2004).

In addition to their function as photon capture pigments in green plants (Taiz & Zeiger 1998), carotenoids also serve as photoprotective agents of the plant photosynthetic centre. By quenching excessive energy from excited chlorophyll they protect the plant cells from damage of the membrane lipids caused by singlet oxygen (Tracewell

et al. 2001). Xanthophylls also undergo reversible enzymatic conversion, known as non-photochemical quenching or xanthophyll cycle, regulating excitation energy in photosystems (Garcia-Plazaola et al. 2007). Moreover, the delocalization of electrons in conjugated bond system enables carotenoid function in free-radical scavenging (El Agamey et al. 2004). The unstable long conjugated molecules of carotenoids are easily attacked by free radicals (Britton 1995) and the delocalized electrons are then easily released and transferred to a radical molecule. At the same time the delocalized electrons may stabilise the newly formed carotenoid radical (Burton & Ingold 1984) and interrupt the propagation of radical chain reaction (Woodall et al. 1997). The products of this reaction can be cleaved and oxidized further (Handelman et al. 1991; Mordi et al. 1993). Moreover, carotenoids may undergo autoxidation and possess prooxidative properties. This type of carotenoid degradation occurs especially in a highly oxidative environment, e.g. in mitochondria, where the highly reactive breakdown products such as aldehydes or epoxides are formed (Siems et al. 2005; Prasain et al. 2005). These molecules may react with amino acids in peptides and proteins, lipids and nucleic acids and thus impair essential cell functions (Siems et al. 2002).

INFORMATION ON HEALTH AND DISEASE IN CAROTENOID-BASED ORNAMENTAL SIGNALS

Parasites cause, by definition, harm to their hosts (Clayton & Moore 1997; Lawrence 2008). This is common to all kinds of parasites including viruses, parasitic bacteria, fungi, protozoa and metazoa. Many parasite infections have a deleterious effect on host health, sometimes even leading to its death. To prevent parasites from inducing disease, i.e. from pathogenicity, animals possess a sophisticated network of defence mechanisms. These include behavioural, structural and immunological components and we can imagine these three respectively as a system of layers protecting the organism in a successive order (see e.g. Hart 1997). First the parasites need to overcome the behavioural defence which includes among others parasite avoidance (Kavaliers et al. 2006), habitat selection (Rohner et al. 2000) and cleaning (Knouft et al. 2003). The body surface then represents a physical barrier for the parasite incursion (Frischknecht 2007). Finally, if parasites eventually succeed in penetrating the host body, the immune system is triggered to eradicate the infection (Wakelin & Apanius 1997).

Despite this efficient system of health protection many parasites have adapted to overcome the host defence. Virtually all animals living in natural conditions harbour at least some parasitic organisms in their bodies although this fact can hardly be observed in wildlife. Due to lowered survival abilities of the diseased animals, the vast majority of the parasite-bearing individuals that we observe suffer only from asymptomatic infections. It is known in birds, for instance, that although significant numbers of individuals harbour avian malaria or coccidian infections most individuals appear healthy (Hörak et al. 2004; Vinkler et al. 2010). Even these healthy-looking individuals are, nevertheless, afflicted by the infection. Parasites reduce the amount of nutrients available to the host organism, and this includes important micronutrients such as carotenoids (see e.g. Ruff et al. 1974; Allen 1992). Besides this direct negative effect of the parasites themselves, the host organism is also harmed by the activity of its own immune system aimed to destroy the parasites (Graham et al. 2005). In the course of the immune response, especially if this response is inflammatory, many host cells are destroyed, which disrupts the integrity of the infected tissues. Mounting an immune response is also costly energetically (Sheldon & Verhulst 1996), as has been demonstrated by the results of many ecological experiments (Moret & Schmid-Hempel 2000; Ots et al. 2001; Poulsen et al. 2002; Martin et al. 2003). This cost may have an important impact on the total energetic budget of an organism, influencing all of its physiology. Thus, mounting an acute immune response compromises other processes, like locomotory activity (Bonneaud et al. 2003; Hörak et al. 2003), moulting (Martin 2005) or reproduction (Råberg et al. 2000; Bonneaud et al. 2003; Garamszegi et al. 2004). On the other hand the trade-off between immune system activity on one side and all other energetically demanding processes on the other may also result in reduced immunity investments (Deerenberg et al. 1997), which may permit an increase in the parasite levels (Richner et al. 1995).

It is thus obvious that any prolonged infection may decrease the individual condition and lower the reproductive success. Individuals, however, markedly vary in their ability to overcome parasite infections. This may result either from their interindividual differences in initial condition (Møller et al. 1998a) or from various immunogenetical traits (see e.g. Apanius et al. 1997; Sommer 2005; Vinkler & Albrecht 2009). Given that parasites may be directly transmitted among individuals it is convenient for both males and females to choose their partners with respect to their health (Sheldon 1993). Moreover, individuals harbouring a disease may be less desirable as mates due to their impaired condition leading to worsened parental care (Iwasa & Pomiankowski 1999; Sasal 2006). Considering also the genetic benefits arising from the mate choice (see 2nd subchapter), it is not surprising that females tend to evaluate the health of their potential mates. Based on our current knowledge, carotenoid-based ornamental traits may provide them with a valuable clue in their choice.

According to the Parasite-mediated sexual selection hypothesis (see 2nd subchapter) females choose their partners based on ornamental traits by which they assess the male's health (Hamilton & Zuk 1982). This hypothesis has two main predictions. Firstly, on an interspecific level, species with more ornamented males are expected to harbour a wider variety of parasites (except for purely acute pathogens) because more intensive parasite-posed evolutionary

pressure promotes parasite-mediated sexual selection. Secondly, within each ornamented species, more ornamented males are supposed to bear fewer parasites than the less ornamented ones, because better health enables expression of highly elaborated ornamentation.

Interspecific analysis performed by Hamilton & Zuk (1982) provided some support for the first prediction and despite much ambiguity in later research (Read & Harvey 1989), at least some of the current evidence speaks for the validity of this hypothesis (although the strength of the relationship is obviously dependent on the taxonomic group in question; Hamilton & Poulin 1997; Møller et al. 1998b). Moreover, the results of many studies carried out on the intraspecific level are consistent with the second assumption given by Hamilton & Zuk (1982), i.e. within population brighter males often tend to be of better health than the drab looking ones. However, as observations of parasite levels may not give convincing results in all investigated species (Hamilton & Poulin 1997), we will focus in the following text on selected examples of several well-studied carotenoid-based-ornamented species, and discuss the evidence in closer detail and with respect to the immune system function.

In Three-spine sticklebacks the hue of male colouration (redness) on the lower jaw is determined by the relative concentration of astaxanthin in skin (Wedekind et al. 1998). It has been shown that this colouration is positively associated with male body condition, health and attractiveness to females (Milinski & Bakker 1990; Kraak et al. 1999; Braithwaite & Barber 2000; Barber et al. 2000). Thus, by their preference for brighter males female Sticklebacks avoid parasitism (Milinski & Bakker 1990). Similar relationships are known in other fish species as well. Carotenoid-based ornamentation in Guppy males also reflects condition and vigour (Nicoletto 1991; Nicoletto 1993; Karino & Haijima 2001). The relationship between colouration and health was verified experimentally by Houde & Torio (1992) who have shown that experimental infection of Guppy males by monogenean parasite *Gyrodactylus tumbulli* decreases the intensity of colouration of the carotenoid-pigmented spots and that the paler males are less attractive to females. Interestingly, in free-living populations of the same species this association was not confirmed by correlation between parasite loads and colour intensity; which however, may be due to other ecological factors influencing the interaction (Martin & Johnsen 2007). In some other fish species, such as Cichlids, this kind of correlation between red carotenoid-based colouration and parasite loads has also been found in field studies (Maan et al. 2006).

As in fish, carotenoid-based colouration was also shown in birds to mirror individual condition (Hill 2002; Badyaev & Duckworth 2003; Casagrande et al. 2007) and health (Møller et al. 2000). Although not every carotenoid-pigmented ornament serves for health signalling (Dale 2000; Fitze et al. 2009), it is well established that parasite infections reduce carotenoid-based ornament elaboration. After parasite infection, the carotenoid levels in blood plasma decrease (Squibb et al. 1971; Ruff et al. 1974; Page et al. 1982) leaving less carotenoids available for the ornament expression (correlation between plasma carotenoid levels and the amount of carotenoids in ornamental structures was reported by Hill et al. 1994; the relationship between plasma carotenoid levels and ornamental colour was shown by Peters et al. 2004b). Thus, for example, it has been shown by Thompson et al. (1997) in House finch that intensities of ectoparasitic feather mite infestation and endoparasitic avian pox viral infection correlate negatively with condition and development of bright male plumage ornamentation during the moulting period. Also in Serin (*Serinus serinus*), ectoparasite load correlated negatively with feather colouration (Figuerola et al. 2003). Moreover, similar results were obtained in various avian species if the health status was assessed based on haematological traits (Figuerola et al. 1999; Saks et al. 2003b). Finally, even experimental manipulations of the parasite burden have revealed identical outcomes. In Blackbird (*Turdus merula*), for instance, experimental infection with *Isoospora* (coccidians) resulted in significant reduction of the bill colouration (Baeta et al. 2008). Equal results although concerning plumage ornaments were obtained by Brawner et al. (2000) and Hill et al. (2004) in House finches experimentally infected with *Isoospora* or *Mycoplasma gallisepticum* and by Hōrak et al. (2004) in *Isoospora* infected Greenfinches (*Carduelis chloris*). The phenomenon of disease-associated decrease in tissue carotenoid levels is collectively referred to as a 'pale bird syndrome' and has also been demonstrated in poultry research in response to various parasite exposures (Tyzzkowski et al. 1991a; Tyzzkowski et al. 1991b; Allen 1992).

Here it is important to mention that not only the activity of parasites reduces ornamentation. Also mere activation of the immune system of the host by non-pathogenic stimuli depletes carotenoids from blood plasma and various tissues (McGraw & Ardia 2003; Koutsos et al. 2003; Alonso-Alvarez et al. 2004; Peters et al. 2004a). As a result, individuals with experimentally manipulated immune function usually show decreased ornament elaboration. This has been proven, for instance, in Blackbirds (Faivre et al. 2003a) and Mallard ducks (*Anas platyrhynchos*; Peters et al. 2004a) stimulated by inoculation with sheep erythrocyte solution or in Zebra finches (*Taeniopygia guttata*) after LPS treatment (Alonso-Alvarez et al. 2004).

Consistently with the predictions of Parasite-mediated sexual selection hypothesis, several pieces of evidence also indicate that highly ornamented males sire healthier offspring. For instance, in Sticklebacks it was shown by Barber et al. (2001) that brighter males father young that are more resistant to cestode parasite *Schistocephalus solidus* infection, potentially due to more intensive mobilisation of leukocytes into the blood stream after infection. Thus, females might, indeed, obtain better genetic material ('good genes') for their offspring by mating with brighter males.

As concluded by Hamilton & Poulin (1997) the Hamilton's and Zuk's hypothesis (Hamilton & Zuk 1982) does

not apply equally well to every taxon and host sexual trait. It is very probable that different evolutionary pressures have shaped the relationship between parasites and host ornamentation making it specific in every host-parasite system. Nevertheless, based on current evidence the role of carotenoid based ornamentation in health signalling in various animal species is indisputable and the mechanism preserving its honesty requires a rigorous explanation.

CAROTENOID AVAILABILITY FOR ORNAMENTAL TISSUES

All animals obtain carotenoids exclusively from their diet (Olson & Owens 1998; Møller et al. 2000). This diet is, however, very diverse. Accordingly, also the amount of carotenoids ingested by different animals is different. The natural carotenoid rich diet may consist of algae in fish and water birds (Karino & Haijima 2004), fruits and seeds are used as a source of carotenoids in many avian herbivores (Hill 2002), while insectivorous species are supplied by carotenoids from invertebrates, especially caterpillars (Eeva et al. 2010). Different species and different individuals within species physiologically vary in their requirements of carotenoid intake, in their carotenoid absorption rates and also in their carotenoid utilisation efficiency (Olson & Owens 1998; Møller et al. 2000; Hughes 2001; Tella et al. 2004). Moreover, also different types of food may significantly vary in the quality and quantity of their carotenoid content, which influences the carotenoid intake (Cohen et al. 2009b). This is also true for seasonal changes in carotenoid availability depending on the presence of a carotenoid-rich diet (Tummeleht et al. 2006). The carotenoid-content of the diet is then mirrored in ornamentation. For instance, lutein and zeaxanthin have been identified as important carotenoid components in the diet of insectivorous birds (McGraw 2006a), and in these species are also often used for ornament expression (McGraw et al. 2003a; Andersson et al. 2007). In the House finch natural carotenoids directly obtained from the food and circulating in blood plasma during the moulting period involve xanthophylls (lutein and zeaxanthin), β -carotene, β -cryptoxanthin, gazaniaxanthin and rubixanthin (McGraw et al. 2001; McGraw & Schuetz 2004). As long-term carotenoid stores are not utilised to colour feathers in birds, only carotenoids obtained during moulting are important for carotenoid-based ornament expression (Brush & Power 1976; Hill 1992). Thus, diet preferences may change seasonally towards a greater preference for carotenoid-rich diet during the moult, when carotenoids are needed to paint newly growing ornamental feathers (Hill 2002). A different situation is in ornamental traits derived from skin or skin derivatives. Here the ornamental traits may undergo rapid changes. It has been shown in kestrels (*Falco tinnunculus*) that carotenoid supplementation in the diet increases carotenoid plasma levels shortly after administration and changes in skin colouration are already visible after 15 days (Casagrande et al. 2007).

It has been found that carotenoid access may limit expression of carotenoid-based ornamentation both in captivity (Brush & Power 1976; Kodric-Brown 1989; Hill 1992; Hill 1994; Karino & Haijima 2004) and in nature (Endler 1983; Grether et al. 1999). Based on this knowledge, the *Carotenoid-limitation hypothesis* was proposed to explain the observed variability in carotenoid-based ornaments. It assumes that only individuals that are able to obtain and retain sufficient amounts of carotenoids may develop brightly coloured ornamentation (Grether et al. 1999). This ability may depend on quality or quantity of food resources available to an individual and hence carotenoid-based colouration might signal the individual foraging abilities (Endler 1980; the idea is called *Foraging ability hypothesis*, see e.g. Lozano 1994 or Perez-Rodriguez 2009). For example, in House finch it has been shown that dietary carotenoids have a direct impact on red breast ornamentation (Hill 1990; Hill 1992; Hill et al. 2002). Similarly, Blount et al. (2002b; 2003) reported that the amount of carotenoids in the diet influences the redness of bill in Zebra finch males, the yellowness of the integument colouration in Lesser black-backed gull females (*Larus fuscus*) and Cote et al. (2010) described the analogous effect of carotenoids on the belly colouration in the common lizard (*Lacerta vivipara*). In Guppies male ornamentation may to a large extent be determined by abilities to forage on a carotenoid-rich diet such as algae (Karino et al. 2007) and in Eurasian Siskin males (*Carduelis spinus*) Senar & Escobar (2002) suggest that the yellow wing stripe may indicate the ability to search for food resources individually. Food preferences for artificially coloured orange and red foods over less colourful options in sticklebacks and guppies may indicate that fish selectively chose potential sources of dietary carotenoids (Rodd et al. 2002; Smith et al. 2004; Grether et al. 2005). In all these cases the colouration may reflect an individual's health because only healthy individuals will obtain sufficient sources to deposit these pigments into highly elaborated ornamentation (Lozano 1994).

Although many ecological studies assume a scarcity of carotenoids available for ornament expression, other studies argue that this may not be always the case (Møller et al. 2000). This throws some doubt on the Carotenoid-limitation hypothesis. If carotenoids are not so rare in the environment, why should they be limited to the ornament expression? Several authors proposed that the limitation may appear in carotenoid absorption, transportation or metabolism (Hill 2000; McGraw et al. 2003b). To utilise dietary carotenoids these first need to be transported through the wall of the gut into metabolically active tissues. Here they are metabolised into molecules utilisable to a particular physiological function or deposition (Chew 1993; Møller et al. 2000; Hill 2002). The main sites of carotenoid metabolism in birds may include liver, small intestine and kidney (Brush 1990) but also the skin, i.e. the tissue where follicles producing ornamental feathers mature (McGraw 2004). Some carotenoids may serve

as precursors to other important compounds. For instance β -carotene is a precursor to vitamin A (Bendich 1989). However, this is a feature of less than 10% of all carotenoids ingested by animals (Chew 1993). In other cases carotenoids themselves are physiologically essential to the organism and are only modified into different forms of carotenoids (Olson and Owens 1998; Palozza et al. 2006).

In some cases, dietary carotenoids may be utilised directly for ornament expression. For example, 4-oxo-carotenoids obtained from aquatic invertebrates (Matsuno 2001) are used by flamingos for feather colouration (Fox 1962; Fox et al. 1970). However, many other carotenoid-based ornaments require for their expression metabolisation of the dietary carotenoids before deposition. After ingestion, natural carotenoids in a vegetable-based diet such as lutein, carotenes, zeaxanthin and cryptoxanthin are metabolically modified through dehydrogenation (lutein and zeaxanthin) to produce yellow canary xanthophylls (Stradi 1998), or undergo 4-oxidation to produce red 4-oxo-carotenoids such as astaxanthin, 4-oxo-rubixanthin, 4-oxo-gazaniaxanthin and cantaxanthin (Stradi 1998; McGraw et al. 2001; McGraw et al. 2002). Carotenoid metabolism is very complex and hence often sensitive to external conditions, such as nutritional changes and pathogen infections (Hill 2002). In House finches it has been shown that nutritional condition and not the access to carotenoids may be reflected by carotenoid-based ornament brightness (Hill & Montgomerie 1994; Hill 2000). The evidence based on experimental treatments inducing nutritional or oxidative stress at the time of moult reveals that metabolic pathways leading to the production of red 4-oxo-carotenoids are particularly costly and sensitive to stress (Hill 2000; Hill 2002). Thus, energetic requirements for carotenoid metabolism might ensure honest signalisation of individual quality.

Contrary to this conclusion, much of the evidence currently available shows that at least some of these processes (e.g. absorption and transportation) are in fact, due to the carotenoids lipid solubility, completely passive and thus costless (Parker 1996; Furr & Clark 1997; Spencer et al. 2006). It has been proposed by McGraw et al. (2005) that the amount of lipids available to bind carotenoids during the phase of their intestinal absorption and transportation may be responsible for the observed effect of the non-carotenoid diet items on carotenoid-based colouration. Nevertheless, even this mechanism of carotenoid intake limitation would maintain the function of carotenoid-based colouration as a signal of the individual foraging abilities.

CAROTENOIDS AND ENHANCEMENT OF IMMUNE RESPONSIVENESS

The most original function of carotenoids in living cells (in bacteria as well as in fungi and animals) is probably to reinforce cell membranes (Vershinin 1999). Although this role has been preserved, carotenoids also acquired some new functions in evolution including light absorption, pigmentation or antioxidant activity. Here we will focus on another role of carotenoids – on their *immunostimulatory* and *immunomodulative* potential which is closely related to health and may be important for our understanding of the signalling function of carotenoids in animal ornamentation.

Carotenoids seem to play an important role in immune system regulation. Various pieces of evidence suggest that carotenoids are potentially able to enhance immune system function, which has been reported in a wide variety of studies performed in humans as well as in different animals like mice, cats, dogs, various birds and fish (Chew 1993; Chew et al. 1996; Chew et al. 2000; Kim et al. 2000a; Kim et al. 2000b; Hughes 2001; McGraw & Ardia 2005). Insufficient intake of carotenoids may increase the susceptibility to a range of infectious diseases and the probability of cancer occurrence (Bendich 1989; Bendich 1991; Chew 1993; Gerster 1993; Hughes 2001). In mammals, dietary carotenoids may increase amounts of lymphocytes in peripheral blood, promote the intensity of allograft rejection, enhance NK cell and neutrophil activity, improve antigen-presenting cell signalling and promote cytokine production (Bendich 1989; Bendich 1991; Chew 1993; Hughes 2001). Some carotenoids, such as β -carotene, perhaps due to its provitamin A function, may also have in immune cells a protective effect against UV radiation and mutagenesis (Bendich 1991; Hughes 2001). Carotenoid supplementation in *in vitro* cell cultures increases the proliferation of lymphocytes in response to mitogens (Chew 1993) and similarly, T and B cells originating from β -carotene- and canthaxanthin-supplemented individuals tend to proliferate more intensively after stimulation (Bendich 1989; Bendich 1991; Chew 1993; Moriguchi et al. 1996). In addition, in birds such as Zebra finches and Greenfinches a positive effect of higher dietary carotenoid (lutein and zeaxanthin) intake on cellular and humoral immunity was shown (McGraw & Ardia 2003; Aguilera & Amat 2007) and identical results were reported also in studies in fish (Grether et al. 2004; Clotfelter et al. 2007). Plasma carotenoid levels tend to correlate positively with the magnitude of the inflammatory immune response (McGraw & Ardia 2003; Perez-Rodriguez et al. 2008). After triggering the immune response, carotenoids are mobilised to blood plasma (Costantini & Dell’Omo 2006), potentially to help to protect tissues; but the plasma carotenoid concentration tends to drop afterwards (McGraw & Ardia 2003; Aguilera & Amat 2007; Perez-Rodriguez et al. 2008). Although it has been suggested that different carotenoid compounds may have a different effect on the immune system (Chew 1993; Chew et al. 1999; Hughes 2001; McGraw & Ardia 2004), not much is known about this variability yet. The immunoenhancing effect of dietary carotenoids may have direct consequences for parasite loads. For instance, it has been shown in Blackbirds that carotenoid supplementation may reduce the *Isospora* parasite replication rate (Baeta et al. 2008) and in Swallows

(*Hirundo rustica*), individuals with higher circulating plasma carotenoid levels had lower levels of immunological markers of disease (Saino et al. 1999).

The effect of carotenoid supplementation on immune function is usually non-significant in healthy organisms in good condition (Hughes 2001) because these are not limited by the carotenoid intake (Møller et al. 2000). Nevertheless, carotenoid accessibility or carotenoid utilisation ability may be limited to an individual, as proposed by the Carotenoid-limitation hypothesis, in case that the health is impaired. Thus, carotenoid-based colouration might signalise individual's health because only healthy individuals would be those who obtain enough sources of these pigments to deposit them into ornamental traits in sufficient levels (Lozano 1994). Better health might correspond to a better immunological state and, indeed, the relationship between levels of the carotenoid-based ornamentation and magnitude of the immune response has been found in various avian species, including Zebra finch, Blackbird and Red-legged partridges (*Alectoris rufa*; Blount et al. 2003; McGraw & Ardia 2003; Faivre et al. 2003a; Faivre et al. 2003b; Alonso-Alvarez et al. 2004; McGraw & Ardia 2005; Perez-Rodriguez et al. 2008). This relationship can be, however, also viewed from a different perspective. The *Carotenoid trade-off hypothesis* proposes that ornamentation competes with the immune function for available sources of carotenoids (Lozano 1994). This hypothesis found much popularity in ecological studies and was incorporated into many hypothetical constructs and models that followed. The principle proposed by this hypothesis is supported, for instance, by experimental work in Fighting fish performed by Clotfelter et al. (2007). In this species variability in body colouration is known, with males ranging from blue to red. Clotfelter et al. (2007) found that redder males feeding on supplemental carotenoids increased both inflammatory immune responsiveness and redness compared to controls. In contrast, carotenoid-supplemented bluer males did not become redder but, instead, mounted the immune responses stronger than either control or redder supplemented fish. These results thus directly support the idea of a trade-off in carotenoid allocation between these two functions which is central to many of the current models of carotenoid-based sexual selection signalling (Hörak & Saks 2003). This view is also supported by the results of studies comparing ornament expression with the immune response magnitude, showing also trade-off between these two (Faivre et al. 2003a; Faivre et al. 2003b; Peters et al. 2004a). But why should immune system compete with ornamentation for the carotenoid sources available? Several hypotheses have been proposed to clarify this eventuality and these are explained in the following paragraphs.

ORNAMENTATION AND THE ANTIOXIDATIVE FUNCTION OF CAROTENOIDS

According to the Carotenoid-limitation hypothesis (see 6th subchapter) is the variability among individuals in sexually selected carotenoid-based ornamental traits preserved by differential availability of carotenoids for ornament deposition. In other words, only individuals with sufficient amounts of carotenoids in the ornamental tissue express the highly elaborated, brightly coloured ornament. As also mentioned in previous subchapters the limitation for carotenoid-ornament expression may be among others the trade-off between ornament expression and immune function. Only healthy individuals will be able to retain enough carotenoids for supreme ornament expression. But why should carotenoids be exhausted by the immune system function? To answer this question we need to take a closer look at the mechanisms of the immune system function and the involvement of carotenoids in it.

The immune system is above all dedicated to elimination of parasite infections. Since some of the most important functional mechanisms of parasite killing in immune cells rely on oxidative processes (as e.g. the oxidative burst, Babior 1984), animal immune defence is extremely vulnerable to oxidative damage (Park et al. 1992; Hughes 2001). Oxidative activity of immune phagocytic cells, such as macrophages or neutrophils may also lead to broader tissue damage (Babior 1984). This oxidative damage arises from release of reactive oxygen species, especially free radicals (Babior 1984). Besides immune activity free radicals and other reactive chemical species may also be generated as by-products of metabolic activity, detoxication action or as a result of damage caused by external factors such UV radiation or pollution (von Schantz et al. 1999; Al Gubory et al. 2010). They cause extensive damage especially to macromolecules such as DNA, proteins and lipids (Babior 1984; von Schantz et al. 1999; Al Gubory et al. 2010). It has been shown that free radical activity may be responsible for health disorders and aging (Coyle & Puttfarcken 1993; Hollan 1995; Beckman & Ames 1998). To protect themselves against the harmful effects of the reactive particles all living organisms possess a complex system of *antioxidative mechanisms* relying on the action of various exogenous or endogenous antioxidant molecules (Beckman & Ames 1998; von Schantz et al. 1999; Perez-Rodriguez 2009). Health of an animal may be severely impaired by the imbalance between the level of tissue oxidation and antioxidative protection – the state termed as *oxidative stress* (Monaghan et al. 2009). It has been found that the antioxidant system capacity of an organism may associate with various life-history traits, including reproduction capacity (Cohen et al. 2008; Cohen et al. 2009a; Al Gubory et al. 2010). Based on the results of many biochemical studies it was proposed that also carotenoids may serve in animal tissues as exogenous antioxidants obtained with diet.

In scientific literature the antioxidative properties of carotenoids are often assumed. It has been evidenced especially in *in vitro* studies that free radicals may be quenched by carotenoids (Foote & Denny 1968; Bendich &

Olson 1989). Consequently, it has been proposed that various carotenoid molecules present in animal tissues, such as β -carotene, canthaxanthin and most of all lycopene, might scavenge free radicals also in living cells and thus play an important role in tissue protection against reactive oxygen species (Burton 1989; Bendich 1989; Bendich 1991; Chew 1993). As carotenoid molecules are predicted to be irreversibly destroyed in this process (Foote & Denny 1968; Vershinin 1999; Kiokias & Gordon 2004) it was proposed by von Schantz et al. (1999) that carotenoid pigmentation may be sensitive to the oxidative stress. Individual's antioxidative capacity would be linked to carotenoid-based colouration because carotenoids bleached by free-radicals cannot be deposited into ornamental traits any more. Based on this assumption a concept of a trade-off between carotenoid utilization in free-radical quenching and their deposition into ornamental tissues has been elaborated (Møller et al. 2000; Alonso-Alvarez et al. 2008). This idea led to a new hypothesis on the physiological mechanism preserving honesty in signalling of individual's health through carotenoid-based ornamentation, a concept currently known as *Antioxidant role hypothesis* (see Perez-Rodriguez 2009).

The model of carotenoid allocation trade-off proposed by the Antioxidant role hypothesis fits much of the evidence currently available. In early observational studies, it has been shown that birds in lower quality urban habitats exhibit less intensively coloured ornaments than animals from habitats of a higher quality in rural areas (see e.g. Eeva et al. 1998; Hørak et al. 2000). Although these differences were attributed to different carotenoid availability in these habitats (Eeva et al. 1998), Møller et al. (2000) proposed that a different pollution rate in the areas of investigation associated with distinct demands on antioxidant capacity may be responsible for the pattern observed. Birds under the increased demands on their antioxidative defence would require more carotenoids to act as antioxidants to maintain their health. Therefore, these individuals would allocate carotenoids from ornamentation to antioxidation and grow less elaborated ornaments. In similar fashion also other experimental outcomes may be viewed, for instance those linking the expression of carotenoid-pigmented traits with immune systems activation (Faivre et al. 2003a), parasite infection (Thompson et al. 1997) or reproduction (Negro et al. 1998). However, these earlier studies did not directly measure the carotenoid concentration and antioxidative capacity of the investigated individuals.

Many of the recent studies, nevertheless, also support the Antioxidant role hypothesis of carotenoid-based signalling of health. For instance, Blount et al. (2002b) showed that supplementary dietary carotenoids increase both colouration and antioxidant capacity of blood plasma in gulls. In Zebra finch the individual resistance to oxidative stress is linked to changes in plasma carotenoid content (Alonso-Alvarez et al. 2004). It was suggested by Bertrand et al. (2006a) that carotenoid availability could modulate the trade-off between resistance to oxidative stress and reproduction in this species. This is consistent with the results reported by Pike et al. (2007a) who showed in their experimental study in Three-spined sticklebacks that under the limitation in dietary carotenoid intake males attempted to maintain their carotenoid-based sexual ornamentation at the expense of body carotenoids, which resulted in their reduced reproductive investments, increased susceptibility to oxidative stress and a shorter lifespan.

Since the maternally transferred carotenoids seem to play an important role in maintaining the egg redox homeostasis as well as in physiology of offspring in the first days after hatching (Blount et al. 2000; Saino et al. 2003a), antioxidant properties of carotenoids were much studied in egg yolk. Carotenoids are allocated by females into the egg yolk in a similar way as into the ornamentation (Blount et al. 2002b) and, thus, also here the trade-off between carotenoid deposition and utilisation in the maternal organism may be predicted (Blount et al. 2000). It has been found, for example, in Lesser black-backed gulls that carotenoid supplementation had a positive effect on both female ornamentation and yolk susceptibility to oxidative stress (Blount et al. 2002a; Blount et al. 2002b).

Although the phenomenon of a trade-off between carotenoid allocation to antioxidative protection and ornamentation became over the years the leading explanation of the mechanism preserving honesty of the health signalling, many questions still remain unresolved. What is the role of hormonal regulation in the process of ornament elaboration? Do carotenoids always act as antioxidants? Could carotenoids also be physiologically risky to the organism (Olson & Owens 1998)? The aim of the following subchapters is to give some new insights into the recent research devoted to answering these riddles.

CAROTENIDS AND TESTOSTERONE: THE OXIDATION HANDICAP HYPOTHESIS

Immune protection is significantly modulated by hormonal signalling. Especially the effects of two steroid hormones, the corticosterone and testosterone, are often discussed in this context, both being considered as immunosuppressive. Indeed, much of the current knowledge supports the idea that corticosterone may lower the actions of the immune system, which most probably corresponds with the adaptive response of an organism to stress (El Lethey et al. 2003; Saino et al. 2003b; Rubolini et al. 2005). Also the immunomodulative effect of testosterone is well supported, though the mechanism of its action is far less clear. Several studies provided evidence that testosterone treatment may impair the immune function of various types as well as the anti-parasite resistance (Peters 2000; Casto et al. 2001; Buchanan et al. 2003; Mougeot et al. 2004; Owen-Ashley et al. 2004; Alonso-

Alvarez et al. 2007). The expression of many ornamental traits is controlled by testosterone, although here an opposite pattern is being observed (see e.g. Moss et al. 1979; Stokkan 1979; Zuk et al. 1995; Peters et al. 2000; Evans et al. 2000; Buchanan et al. 2003). Based on the handicap principle proposed by Zahavi (1975) and the evidence of the kind mentioned above, Folstad & Karter (1992) proposed their *Immunocompetence handicap hypothesis* of sexual signalling through ornamental traits. According to their view testosterone may act as a 'double-edge sword' promoting ornamentation on one hand and suppressing parasite resistance on the other. This would lead to a trade-off between immunity and ornamentation and only high quality individuals in which the costs for parasite resistance are low would be able to preserve their health despite the handicap caused by high levels of testosterone and express highly elaborated ornaments.

The proposal of the Immunocompetence handicap hypothesis induced much debate concerning the associations among immune system, endocrine system and ornamentation. To refute objections against the evolutionary stability of the mechanism (see e.g. Hill 2002), Wedekind & Folstad (1994) suggested that the testosterone only mediates the necessary condition-dependent energetic trade-off between immunity and ornamentation (see also Saino et al. 1995; Møller 1995; Mougeot et al. 2004). Involvement of energetic costs and individual condition into this relationship received much support. For instance, Hasselquist et al. (1999), Mougeot et al. (2004) and Owen-Ashley et al. (2004) provided evidence linking higher testosterone levels to decrease in condition-related traits such as body weight or fat stores. This interpretation might also help to explain the somewhat inconsistent evidence for the immunosuppressive effect of testosterone (see e.g. Saino et al. 1995; Hasselquist et al. 1999; Evans et al. 2000; Westneat et al. 2003). Moreover, the actual effect of testosterone on immunity might be in this case mediated by corticosterone (Møller & Saino 1994; Møller 1995), which was supported by the results of Duckworth et al. (2001) in House finch, Buchanan et al. (2003) and Evans et al. (2000) in House sparrow (*Passer domesticus*) and Owen-Ashley et al. (2004) in Song sparrow (*Melospiza melodia*). In fact, the testosterone effect may be completely omitted from the relationship in some cases as it has been shown that carotenoid-based ornamentation may be directly influenced by corticosterone itself (Fitze et al. 2009; Cote et al. 2010).

Besides its immunomodulative and ornament-enhancing potential testosterone signalling also influences the oxidative balance of an organism. It has been shown in several studies that testosterone treatment may depress resistance to oxidative stress, possibly through its effect on metabolic rates (Wikelski et al. 1999; Buchanan et al. 2001; Alonso-Alvarez et al. 2007). This fact is central to the *Oxidation handicap hypothesis*, according to which testosterone mediates the trade-off between ornament expression and free-radical disposal (Alonso-Alvarez et al. 2007; Alonso-Alvarez et al. 2008; see also left side of Figure 3). Based on this hypothesis, Peters (2007) recently proposed a comprehensive model of the physiological interactions behind carotenoid-based signalling. It assumes a trade-off in carotenoid allocation between immunity and sexual signalling under the control of testosterone. Also here the double effect of testosterone is suggested. On one hand testosterone increases the susceptibility to oxidative stress, decreasing also the immune capacity of an organism, while on the other hand elevated testosterone concentrations make carotenoids available for the ornament expression (Blas et al. 2006; McGraw et al. 2006; McGraw 2006b; but see Alonso-Alvarez et al. 2008). This model underlined that the carotenoids acting as antioxidants might compensate for the immunosuppressive effect of testosterone. In other words, the hypothesis proposed by Peters (2007) suggests that the immune system competes with ornaments for the available pool of immunostimulating carotenoids; therefore, ornament elaboration is expected to be determined by the balance between the positive effect of testosterone on the bioavailability of carotenoids, and the negative effect of testosterone on the oxidative state and immunity. Consistently with this model, Kurtz et al. (2007) found in an experimental study in Sticklebacks that males' testosterone levels correlate positively with ornamentation but negatively with immune responsiveness and that there is a trend for fish with high testosterone levels to suffer more from oxidative stress. Alonso-Alvarez et al. (2008) found in Red-legged partridges that testosterone treatment releases carotenoids into plasma, but in this case this had no positive effect on the carotenoid-based ornamentation. On the contrary, testosterone-treated males were pale in ornamentation but they did not suffer from oxidative stress. This led the authors to conclude that the trade-off was resolved by reducing ornamental colouration in favour of increased carotenoid availability for the protection against oxidative damage. These results show that the model proposed by Peters (2007) is a valuable tool to explain much of the variance in honest health signalling through carotenoid-based ornamental traits. Following subchapters, nevertheless, aim at providing some evidence contradicting this view.

RED HERRING IN THE CAROTENOID-BASED-ORNAMENTATION RESEARCH?

Many of the relationships described in the model proposed by Peters (2007) were supported by recent research. However, some other current findings contradict the predictions made by this model. Most importantly, the carotenoid antioxidant activity is nowadays widely discussed (Britton 1995; Hughes 2001; Hartley & Kennedy 2004; Palozza et al. 2006; Tummeleht et al. 2006; Hōrak et al. 2007; Isaksson et al. 2007; Perez-Rodriguez et al. 2008; Perez-Rodriguez 2009). In *in vitro* systems the antioxidant capacity of carotenoids is often tested in pure solutions and at

concentrations across the human physiological range where, indeed, carotenoids show antioxidative activity (Foote & Denny 1968; Burton 1989; Bendich 1989). The results from several studies, nevertheless, indicate that at higher carotenoid and oxygen concentrations carotenoids may lose their antioxidant function and even cause prooxidation (Burton & Ingold 1984; Young & Lowe 2001; Krinsky 2001; El Agamey et al. 2004). Also the behaviour of carotenoids *in vivo* may be quite different from their activity *in vitro* (Young & Lowe 2001). In animal bodies carotenoids do not form pure solutions but they become involved in interactions with many other molecules, some of which are other antioxidants (Hartley & Kennedy 2004; Perez-Rodriguez et al. 2008; Costantini & Møller 2008; Monaghan et al. 2009; Cohen & McGraw 2009). These antioxidants often act synergistically (Young & Lowe 2001; Liu et al. 2004; Jeon et al. 2008; Monaghan et al. 2009; Cohen & McGraw 2009; Perez-Rodriguez 2009). Therefore the antioxidative effect of carotenoids may depend on their interaction with other dietary-obtained antioxidative molecules, such as vitamin C and E (Sies 1993; Brigelius-Flohe & Traber 1999; Liu et al. 2004; Traber & Atkinson 2007), or with endogenously produced antioxidative components (Murphy & Sies 1991; von Schantz et al. 1999).

The lack of strong evidence supporting the prominent antioxidative function of carotenoids was also recognised in the research of carotenoid-based health signalling (Costantini & Møller 2008; Perez-Rodriguez 2009). Some experimental studies failed to find any association between carotenoid supplementation, mobilisation and the protection against oxidative stress (Isaksson et al. 2007; Costantini et al. 2007b; Isaksson & Andersson 2008), providing evidence inconsistent with the current paradigm. These results are, however, in good concordance with the alternative '*Red herring*' hypothesis proposed by Hartley & Kennedy (2004). These authors assume that colourful carotenoid-based ornaments may not signal their own presence but rather sufficient levels of other, colourless antioxidants in the organism. Carotenoids are brightly coloured but they are also sensitive to oxidation and cleavage by reactive oxygen species. The cleavage products have significantly altered chemical and biophysical features (El Agamey et al. 2004). By no means can these act as antioxidants but even more importantly they lack any colour. On the other hand, primarily colourless substances like vitamins C and E or antioxidant enzymes are probably important for protecting tissues against reactive oxygen species. Hartley & Kennedy (2004) therefore assume that these non-coloured antioxidants are responsible for carotenoid protection against potential cleavage associated with bleaching, which preserves sufficient levels of carotenoids for the expression of carotenoid-based ornaments. The authors call this the '*red herring*' of the antioxidant story because this mechanism enables honest signalling of non-colourful antioxidants based on deposition of bright carotenoids. In individuals with impaired health, oxidative stress may take place. Under oxidative stress colourful carotenoids are degraded into colourless cleavage products with no effect on ornament elaboration. In concordance with this hypothesis, non-colourful dietary antioxidants have been recently shown to improve the elaboration of carotenoid-based ornaments in birds and fish (Bertrand et al. 2006b; Pike et al. 2007b; Perez et al. 2008).

CAROTENOID MAINTENANCE HANDICAP

As discussed in the previous subchapters some recent findings contradict the Carotenoid antioxidant role hypothesis (Isaksson et al. 2007; Isaksson & Andersson 2008; Costantini & Møller 2008; Perez-Rodriguez 2009) and even the harmful effect of carotenoids on animal health was proposed (Hartley & Kennedy 2004; El Agamey et al. 2004; Zahavi 2007). Carotenoids often used for ornament expression, such as lutein and zeaxanthin (McGraw et al. 2003a; Andersson et al. 2007), may under certain physiological conditions change their behaviour and increase rather than decrease the risk of oxidative stress (Burton & Ingold 1984; El Agamey et al. 2004; Siems et al. 2005; Rao & Rao 2007; Kalariya et al. 2008; see also Zahavi 2007). This transition between the antioxidative and prooxidative effect of carotenoids is probably dependent on carotenoid and oxygen concentrations (Burton & Ingold 1984; Edge & Truscott 1997; Palozza 1998; Salerno et al. 2005). As mentioned in the 4th subchapter the long aliphatic chain of carotenoids is vulnerable to cleavage by free radicals and other reactive species. As a result highly reactive apo-carotenals are created. In the organism these molecules cause severe cytotoxicity (Siems et al. 2000; Hurst et al. 2005; Alija et al. 2006; Yeh & Wu 2006) and inhibit growth, promote apoptosis and cause immunosuppression (Nara et al. 2001; Nagao 2004; Salerno et al. 2005; Kalariya et al. 2008). Siems et al. (2002) also demonstrated that severe tissue damage may arise from the presence of carotenoids during the macrophage respiratory burst in inflammation. All this evidence suggests that carotenoid maintenance in tissues exposed to high oxidative stress may be hazardous. Hence, carotenoids may be indeed risky as proposed already by Olson & Owens (1998). To incorporate this fact into the current paradigm Vinkler & Albrecht (2010) recently proposed a new hypothesis taking into account the non-carotenoid antioxidative reserves of the organism. Their *Carotenoid maintenance handicap hypothesis* assume that honesty of the carotenoid-based signalisation may be preserved by the balance between the positive physiological and antioxidative function of carotenoids and the detrimental effect of non-colourful carotenoid cleavage products (left vs. right side in Figure 3). This balance would depend on available non-carotenoid antioxidant reserves: when these are low, carotenoids are preferably degraded. The role of testosterone in this relationship is supposed to be ambiguous. On the one hand it is ornamentation-enhancing while on the other one it simultaneously increases oxidative stress leading to the reduction of antioxidant reserves and eventually to carotenoid cleavage. Hence, testosterone would promote the handicap arising from the carotenoid

maintenance. According to this model individuals are expected to optimize rather than maximize the carotenoid intake because maximal intake potentially allowing highest level of the ornament expression also increases the risk of intoxication with carotenoid cleavage products. Only high quality individuals in a prime antioxidative state could afford the maintenance of high testosterone and carotenoid levels in their bodies. Any low-quality males are prevented from elaborated ornamentation by the risk of intoxication without any positive effect on ornament colour enhancement.

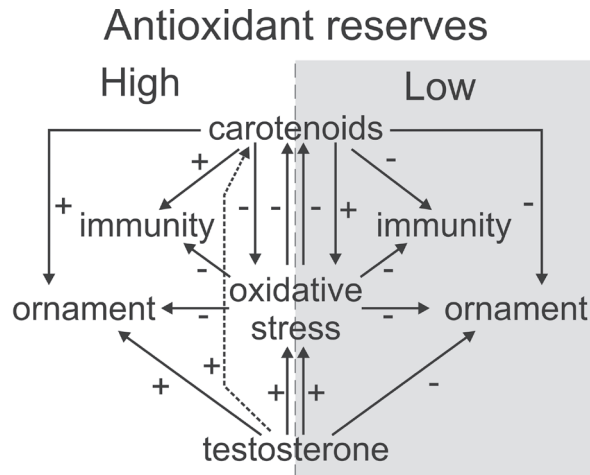


Figure 3. Carotenoid maintenance handicap hypothesis: Relationship between testosterone and carotenoids mediated by oxidative stress under conditions of high and low antioxidant reserves proposed by Vinkler & Albrecht (2010). The left side is consistent with Oxidation handicap hypothesis and the model proposed by Peters (2007). Plus and minus signs indicate predicted positive and negative associations, the dashed line represents effect of testosterone on carotenoid plasma concentrations.

The Carotenoid maintenance handicap hypothesis is based on four predictions (Vinkler & Albrecht 2010): (1) maximal elaboration of the carotenoid-based sexual ornaments is preferred by the signal recipient; (2) the handicap caused by formation of the carotenoids cleavage products may influence the overall physiology of the individual or at least the expression of the ornament; (3) the frequency of carotenoid cleavage is dependent on the body reserves of other, protective antioxidants. (4) testosterone promotes ornamentation but at the same time lowers the protection of carotenoids by other antioxidative molecules.

Current evidence shows that all four of these points may be often met in animals. Firstly, in the mate choice of many species females, indeed, positively select males with maximally elaborated carotenoid-based ornaments (see e.g. Hill 2006). Secondly, as mentioned above, carotenoids tend to become cleaved under specific conditions into cleavage products toxic to animal tissues. This is probably true even for the carotenoids most abundantly present in ornamental traits, i.e. lutein and zeaxanthin (Prasain et al. 2005; Kalariya et al. 2008). Although no specific research has been conducted to investigate the relationship between carotenoid cleavage and ornament expression, the results of several studies suggest that carotenoid supplementation may significantly increase the abundance of the carotenoid cleavage products in animal tissues (Khachik et al. 1997; Prasain et al. 2005; Khachik et al. 2006; Ho et al. 2007). Negative effects of carotenoid supplementation on body weight (Costantini et al. 2007a), tissue integrity (Welch et al. 1999) and specific immune functions related to the oxidative burst (McGraw & Klasing 2006, see also McGraw & Ardia 2007) are also known. Thirdly, also the association between non-colourful antioxidants, carotenoid cleavage and ornamentation has been partially supported by evidence. It is known from *in vitro* studies that non-colourful antioxidants (such as vitamin C) may protect carotenoids from acquiring prooxidative potential (El Agamey et al. 2004). This in turn, might have influence upon the ornament elaboration. For instance, Bertrand et al. (2006b) showed that food supplementation with non-colourful antioxidant melatonin significantly enhances the positive effect of dietary carotenoids on bill colouration in Zebra finches. Similar results were reported also from studies in fish (Pike et al. 2007b) and in gulls (Perez et al. 2008). Finally, several recent studies shed some light on the role of testosterone in regulation of carotenoid physiology. In Red-legged partridge and Zebra finch it has been found that testosterone treatment increases the carotenoid levels in blood plasma (Blas et al. 2006; McGraw et al. 2006; Alonso-Alvarez et al. 2008). These carotenoids are then available for ornament expression. However, another piece of evidence from the same two species suggests that higher testosterone levels may increase the oxidative burden and potentially lead to oxidative stress (Alonso-Alvarez et al. 2007; Alonso-Alvarez et al. 2008; Mougeot et al. 2009). The effect of testosterone on physiological processes influencing ornamentation is therefore equivocal (see also McGraw 2006b).

According to the Carotenoid maintenance handicap hypothesis individuals should optimise and not maximise the levels of carotenoids present in the organism. Hence, one can predict the existence of a certain level in carotenoid supplementation experiments above which higher carotenoid doses in the diet do not affect the plasma levels of these pigments. A plateau like this has been found in fish (Doolan et al. 2009) and birds (Alonso-Alvarez et al. 2004) as

well as in mammals (Mathews-Roth 1990). As shown by Alonso-Alvarez et al. (2004) the plateau level drops down after stimulating the immune system, despite sufficient carotenoid intake. If this plateau really is the point at which carotenoids are in equilibrium with other antioxidative substances, however, remains uncertain. Some support to this idea is given by the results of Koutsos et al. (2003) who show that the decrease in carotenoid levels following immune system stimulation is higher in animals under high dose supplementation. This is in contrast to what could be expected if carotenoids promoted tissue protection and is consistent with the idea of a tissue carotenoid balance.

Further experimental work is highly required to test the Carotenoid maintenance handicap hypothesis. Nevertheless, at the current stage of knowledge it seems that this hypothesis might offer a valuable view on the mechanisms preserving honesty of the carotenoid-based health signalling. By its emphasise on optimisation instead of maximisation of the carotenoid intake, it may help to explain some of the currently puzzling results, such as why testosterone treatment did not affect colouration in some studies (Alonso-Alvarez et al. 2007) while in others such a treatment even reduced ornamentation in carotenoid-supplemented individuals (Stoehr & Hill 2001; Siitari et al. 2007).

CONCLUSION

Although the concept of sexual selection is known for more than a hundred years (Darwin 1871), the research devoted to investigation of mechanisms behind the carotenoid-based signalling in mate choice has undergone a tremendous advance only recently. The importance of the carotenoid-pigmented ornaments for animal communication in sexual selection has been recognised and the impact of parasites on the ornament expression suggested (Andersson 1994). As proposed by the Parasite-mediated sexual selection hypothesis (Hamilton & Zuk 1982) ornamental traits might offer the clue to judge individual level of parasite burden as well as species one. On the basis of ornamentation females could assess the heritable resistance to parasite infections in their mating partners and thus improve the quality of their offspring. Much is presently known about the associations between carotenoid-based ornamentation on one side and parasite loads and immune function on the other side. Complex framework of theories has been developed to explain the observed interactions, including limitations and trade-offs revealed to govern the ornament expression. Indeed, as shown by the integrative model proposed by Peters (2007), most of our current knowledge concerning the ornament expression fits well. Carotenoid allocation to ornamental tissue appears to be limited by other sinks of their utilisation, mainly the immune activity and antioxidative defence while the mobilisation of carotenoids to the bloodstream is under the hormonal regulation of testosterone. Testosterone may improve the availability of carotenoids for ornamental traits but at the same time it also increases the risk of oxidative stress impairing the ornament expression. Only healthy individuals might afford to allocate carotenoids from immunological processes and antioxidative defence to ornamentation preserving honesty of the health signalling.

However, despite current intensive research effort in this field, many aspects of the health signalisation via ornamental traits still remain unresolved. Most interestingly and perhaps unexpectedly, it has been recently questioned whether carotenoids really act as efficient antioxidants in animal bodies (Perez-Rodriguez 2009) and, moreover, even the detrimental effect of carotenoid supplementation on health has been reported (Heinonen et al. 1994; Omenn et al. 1996; Welch et al. 1999; Costantini et al. 2007a). This evidence is inconsistent with the present paradigm on the honesty of carotenoid-based signalisation of health status as described above and has lead several authors to propose new hypotheses on this topic. As suggested by Hartley & Kennedy (2004), carotenoids might in fact signalise the availability of non-colourful antioxidants. By adding the assumption of harmfulness of carotenoid cleavage products and the handicap principle concept regulated by testosterone, Vinkler & Albrecht (2010) formulated the hypothesis of Carotenoid maintenance handicap. This hypothesis proposes that under insufficient antioxidant reserves carotenoids remain unprotected to oxidative cleavage and hence a physiological trade-off may arise between maintenance of carotenoids for ornament expression and their removal from the body. Testosterone may promote oxidative stress and thus lower the antioxidant reserves, but it also enhances the carotenoid availability. The presence of high levels of carotenoids is required for highly elaborated ornament expression. Therefore a handicap may result from carotenoid maintenance ensuring that the brightly coloured ornamentation is affordable only to high-quality individuals. This mechanism might preserve the honesty of the health signalling via carotenoid-based ornamentation.

Nevertheless, this hypothesis requires much testing to verify its assumptions. First, there is no evidence available at present showing that in animals with low antioxidant reserves the supplementary carotenoids impair both ornament expression and immune function. As proposed by Vinkler & Albrecht (2010) reasonably strong evidence confirming the existence of these relationships would disprove the current paradigm and support the Carotenoid maintenance handicap hypothesis. To manipulate the antioxidant reserves, the treatment groups should differ in more aspects than simply the presence or absence of supplementary carotenoids. Antioxidative molecules such as vitamins C and E (Liu et al. 2004) or even supplementary proteins (to increase endogenous production of free-radical scavenging enzymes; von Schantz et al. 1999) should be included. Little is also known about the potential adverse effect of testosterone on ornament expression. In particular, the increase in carotenoid bioavailability after testosterone

supplementation requires further attention as the destiny of the carotenoids that emerge in blood plasma after high-dose testosterone manipulation (Blas et al. 2006) is unclear. Although these carotenoids may be deposited into ornamentation, they may be sometimes removed from the ornamental tissue (see Alonso-Alvarez et al. 2008) and possibly even from the body. Worth examining is also the effect of testosterone on carotenoid cleavage. Finally, more evidence is required to the cytotoxic properties of carotenoid cleavage products and their prooxidative potential. In tissues with low antioxidative capacity, increased oxidative damage caused by the release of reactive oxygen species is predicted when carotenoids increase only according to the Carotenoid maintenance handicap hypothesis and not according to the present paradigm. In this case, single test measurements may be, however, problematic to make clear estimates of the tissue antioxidative capacity or oxidative state (Monaghan et al. 2009; Cohen & McGraw 2009; Perez-Rodriguez 2009).

Testing hypotheses that form the theory explaining the carotenoid-based signalling of health may give us valuable insights into the mechanisms governing the mate choice, evolution of ornamentation and speciation. Research in this field is also of a principal importance for our understanding of the host-parasite interactions, their ecology and evolution. Hence, our knowledge about the carotenoid-based ornamentation gains application potential as well. Understanding the mechanisms ensuring the honesty of health signalisation through carotenoid-based secondary sexual traits in nature may give us in many cases a clue to sustainable game and wildlife management, especially when artificial selection in captivity is being adopted. This issue is particularly meaningful in commercial breeding nowadays when usage of many antibiotics and other drugs is discouraged in leading countries either by legislation or public opinion. Selection promoting natural resistance to diseases may therefore represent in many species a suitable alternative (Kaiser 2010). In case of their rightness, the implications of several hypotheses such as for example the Carotenoid maintenance handicap hypothesis may have a significant impact on current theories on animal diet. Carotenoids represent one of the basic micronutrients and their positive effect on animal health is often assumed. If, however, carotenoids may be in particular ecological contexts harmful we should be aware of this fact when planning the guidelines to animal diet in captivity and breeding. Taken all together, we believe that investigation of the signal function of carotenoid-based ornaments with respect to animal health may be a rewarding approach both to improving our knowledge of animal ecology and evolution and to utilising this knowledge in practice.

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Paper IX

Title: Handling ‘immunocompetence’ in ornithological studies: Do we operate with confused terms?

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Abstract

‘Immunocompetence’ is a term used in avian immunoeology to refer to the ability of an individual to overcome potential parasite infections. However, there are multiple ecological definitions of this term currently used and all of them are rather liberal in immunological terms. This prevents much of the potential intellectual interchange between ornithology and immunology, which decelerates the development of immunoeology as a scientific discipline. We therefore highlight that the term should be handled with care. In any individual host-parasite interrelationship the demands on host immunity are distinct and thus also the measurements of immunity in any particular case should be aimed differently. Although ornithologists often aspire to obtain a single variable for immunity in their research, due to the enormous diversity of parasites possessing the ability to infect the host, there is no single value for anti-parasite resistance, i.e. no overall ‘immunocompetence’ *per se* exists. We propose to use more rigorous terminology, consistent with the one used in classical immunology. The term ‘immunocompetence’ (defined as the ability to produce anti-parasite or anti-antigen immune response) should be used as 0/1 character. The magnitude of a particular immune response (i.e. a continuous quantitative trait) should be referred to as ‘immune responsiveness’. Most importantly, both terms should always be used only with respect to a certain parasite taxon or antigen studied as otherwise they lose their explanatory value.

Key words

Ecological immunology, ecoimmunology, immune responsiveness, immunity, immunocompetence, immunoeology, PHA, phytohaemagglutinin

Article

Current avian research often focuses on host-parasite interactions and investigation of ecological aspects of immunity, spawning immunoecology as an emerging scientific discipline (Ardia and Schat 2008). Aiming to describe the differences observed in immune function among individuals, ornithologists in many cases deal with the term ‘immunocompetence’, which is assumed to be a hidden and more general trait detectable based on the immunological test adopted (see e.g. Martin-Vivaldi et al. 2009; Moreno-Rueda 2010; Jacquin et al. 2011). Despite a well-developed theoretical background in ecology and a substantial amount of data already collected the immunological aspects of the problem in question still often tend to be oversimplified. Thanks to the present rapid advance in immunology, immunity (the ability to resist disease; Lawrence 2008; Cruse and Lewis 2009) is no more perceived as a black box but is now understood as a vivid system of interlaced mechanisms of a diverse function. However, ecologists have always found a bit difficult to follow the most recent discoveries in immunological research and until recently not many immunologists have been open to join ecological studies (Seed 1993). Although this slowly starts to change (see Pedersen and Babayan 2011), the cooperative effort of immunologists and avian ecologists still remains uncommon. This may partially result from different way of thinking and distinct terminology in both fields continuously leading to much misunderstanding. We therefore assume that highlighting these differences and uniting the language may be crucial to build a bridge for sharing the knowledge between these two disciplines, which could be eventually beneficial to both of them (Seed 1993). A case in point of this contribution is the manner of handling the term ‘immunocompetence’, a current cornerstone of avian immunoecological investigation.

What is ‘immunocompetence’?

Since the seminal article by Folstad and Karter (1992) ‘immunocompetence’ has become a pivotal term in immunoecology which has, nonetheless, never been defined properly. Folstad and Karter (1992) themselves did not formulate any definition of ‘immunocompetence’. Later, Norris and Evans (2000) defined ‘immunocompetence’ as the “ability of host to prevent or control infection by pathogens or parasites” (also see Beldomenico and Begon 2010). However, the host organisms are attacked by a wide variety of parasites in an unpredictable set (Sheldon and Verhulst 1996; Schulenburg et al. 2009). If no particular parasite is mentioned (which is usually the case in the current immunoecological literature), we cannot simply know what an author of a study meant by this term: ‘immunocompetence’ to what is being reported. Of course, he or she might assume the average value of immunity to all potential parasites. This interpretation of the definition of ‘immunocompetence’ would seem practically useful if shared levels of host resistance to different parasites existed. Immunoecology has much focused on the energetic balance (a trade-off) between immune system activity and other physiological functions, such as reproduction (Sheldon and Verhulst 1996). If the immune function is viewed only from this energy-allocation perspective, then, indeed, shared levels of resistance may be expected and described as ‘immunocompetence’. However, although the amount of resources available for immunity is undoubtedly a significant factor determining the

potential magnitude of an immune response against pathogen (and this was important to realise), environmental factors alone are not enough to explain the rules governing host-parasite interactions. Other trade-offs may exist due to specialisation of immunological pathways (for instance Th1 vs. Th2 vs. Th17; Kaiser and Staheli 2008), evolutionary adaptations of species (Blount et al. 2003) and immunity-immunopathology balance (Graham et al. 2005). The way of resolving these trade-offs is equally important and at least partially genetically encoded. Thus, host individual genotype, together with environment, is responsible for the outcome of interaction between host immunological polymorphism and intruding parasite variability (Sheldon and Verhulst 1996; Drury 2010). Here we find wide evidence for lock-and-key mechanism in the interaction (see, e.g., Sommer 2005; Vinkler and Albrecht 2009). A host genotype beneficial in fight against one particular parasite species may be disadvantageous against another. This is because the efficiency of a particular immunological mechanism in the defence against a certain parasite infection depends largely on the specific properties of the parasite in question (Wakelin and Apanius 1997) and the host capability to mobilise this specific immunological mechanism is determined or co-determined by its genotype (Sommer 2005; Vinkler and Albrecht 2009; Lazzaro and Little 2009). Although in some cases there may be some congruence in magnitudes of responses based on different immunological mechanisms (Saks et al. 2003), this is not the case in general (Gonzalez et al. 1999; Blount et al. 2003; McGraw and Ardia 2005; Saks et al. 2006; Bonato et al. 2009; reviewed in Adamo 2004 and Drury 2010). Hence, the relative host ability to control infections caused by different parasites may vary (Viney et al. 2005) and therefore also the existence of overall ‘immunocompetence’ *per se* is highly questionable.

In practice it is rather problematic to experimentally test the host ability to prevent parasite infections in its all-inclusive complexity. This lead ecologists to study the activity of individual immunological mechanisms instead (see, e.g., Norris and Evans 2000 or Drury 2010) resulting in modified definition of ‘immunocompetence’ as a “magnitude and effectiveness of an animal’s immune response” (Adamo 2004). The shortcoming of this view is, however, that we do not know which immune mechanism is crucial for the survival of the animal and there is only limited congruence between different immune responses and anti-parasite resistance (see, e.g., Owen and Clayton 2007). Moreover, we cannot also easily assume that greater magnitude of the immune response is better for the anti-parasite defence (Viney et al. 2005).

Contrary to this ecologists’ perspective, ‘immunocompetence’ is in the viewpoint of classical immunology comprehended much more as “the capacity to respond immunologically to antigen stimulation” (Lawrence 2008; Cruse and Lewis 2009) or the ability to produce a normal-type immune response (see, e.g., Fellah et al. 2008). Thus, it refers to a current specific capacity to respond to a particular stimulus. In this meaning ‘immunocompetence’ is a 0/1 character which is not measurable in scale. An organism is either immunocompetent, if it is able to trigger the immune response, or immunocompromised, if there is no immune response or the immune response is aberrantly low (which is usually not the case in free-living animals). This is not just an expressional difference. Let us give an example. If the bird A is able to respond strongly to a particular viral vaccine and less

strongly to a certain bacterial one while in the bird B the situation is opposite, then both individuals are immunocompetent according to the ‘immunological’ perspective (mirrored, e.g., in definition given by Lawrence 2008) while by the ‘ecological’ definitions (e.g. by Norris and Evans 2000 or by Adamo 2004) the evidence is equivocal. However, the second interpretation is rather incorrect as it neglects to account for the difference in the source of stimulus as well as for the fact of optimisation of the immune response magnitude. Thus ‘immunocompetence’ seems to be ill defined in ecological studies and terms more precisely describing the observed results are required.

‘Immunocompetence’ and the phytohemagglutinin skin-swelling test

The term ‘immunocompetence’ is most commonly used in avian immunoecology to describe the results of the phytohemagglutinin (PHA) skin-swelling test (at present almost one third of all ecological articles dealing with ‘immunocompetence’ at Web of Science refer to PHA). Given that the functional nature of the immunological mechanisms behind the test remains unresolved and obviously very complex (Martin et al. 2006; Kennedy and Nager 2006; Owen and Clayton 2007; Vinkler et al. 2010), the term ‘immunocompetence’ seems to be a suitable term for this purpose. Nevertheless, from the immunological standpoint it is rather misleading. PHA is not a natural antigen occurring in any parasite-derived structures (Licastro et al. 1993) and further investigation is needed to verify whether the test reflects resistance to any particular parasite types (Gonzalez et al. 1999; Saks et al. 2006; Owen and Clayton 2007). More likely than giving us a tool to judge any general ‘immunocompetence’, the results of the test may give us a clue to predict the pro-inflammatory capacity of the individual (Vinkler et al. 2010). Based on the knowledge of the efficiency of inflammation in clearance of specific parasite types, this information can be later used to rigorously test the differential resistance to particular parasite taxa naturally occurring in the environment of their host. However, as the resistance of an individual to other parasite taxa may be (and most possibly is) different, no general ‘immunocompetence’ is measured by the PHA test.

Conclusion

To conclude, we would recommend avian ecologists to use the term ‘immunocompetence’ (defined as the ability to produce anti-parasite or anti-antigen immune response) as a 0/1 character and only with respect to a certain parasite taxon or antigen studied. On the contrary, the magnitude of a particular type of an immune response to specific antigen (i.e. a continuous quantitative trait) should be better referred as ‘immune responsiveness’. We believe that adoption of more precise terminology in avian ecological studies may be highly beneficial for interdisciplinary information interchange between ecologists and immunologists which is needed to maintain immunoecology as an up-to-date science discipline.

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Paper X

Title:

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

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Abstract

The phytohaemagglutinin (PHA) skin-swelling test is one of the most widely used methods for cell-mediated immunity measurement in immunoecology. Despite reasonable effort devoted to investigation of 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. In this study we therefore examined the linkage between several condition-dependent traits including red plumage ornamental colouration and the PHA-induced immune responsiveness in scarlet rosefinch (*Carpodacus erythrinus*) males. Our results show two important aspects of the PHA-induced inflammation in this species. Firstly, the histological analysis has revealed that the swelling response is dependent on basophil activity. Secondly, being associated with individual size, carotenoid-based ornamental colouration and a ptilochronological marker of condition in the time of moulting (a mean growth bar width), the PHA-response magnitude (increase in patagium thickness) mirrors the long-term quality of the individual. The positive linkage between the individual size or mean growth

bar width and the PHA responsiveness suggests association of the magnitude of the response to individual metabolic rate. However, as the magnitude of the response is also negatively related to ornament saturation and positively to ornament brightness, our data indicate stronger responsiveness in inferior males. To our knowledge this study is the first to show the negative association between carotenoid-based plumage colouration and the magnitude of the PHA-induced immune response and also the first to investigate linkage between PHA responsiveness and a ptilochronological trait.

Key words: Condition, ecological immunology, ecoimmunology, female mate choice, health signalling, immunocompetence, ornamentation, phytohemagglutinin, sexual selection

Article

Many ornamental traits important in mate choice are supposed to signalise individual health and vigour (Andersson 1994; Vinkler & Albrecht 2010). Hence, there is no surprise that behavioural ecologists interested in sexual selection often concentrate on investigation of ecological aspects of health state contributing thus to the fast development of immunoeecology as a research discipline. Among the immunological methods utilised in behavioural studies only a few received general popularity over the past two decades. The phytohaemagglutinin skin-swelling test is undoubtedly one of these techniques widely used in birds and mammals (Kennedy & Nager 2006; de Bellocq et al. 2007; Ardia & Schat 2008). Until these days a large amount of potentially valuable data has been gained on the basis of this particular method. For instance, adopting the PHA skin-swelling test much effort has been 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 phytohaemagglutinin (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 period after the PHA treatment minus the tissue thickness before the PHA injection. Although the results of the PHA skin-swelling test are commonly referred as measurements 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)), present data suggest that this response might be in fact a non-specific inflammation without T cell dominant involvement (Martin et al. 2006; Sarv & Hõrak 2009; Vinkler et al. 2010a). No matter what the actual immunological mechanism behind the response is, the intensity of the response has been evidenced to be heritable in some species (Bonneaud et al. 2009), while not in others (Christe et al. 2000). This is in concordance with the 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 co-determined traits should be at least partially stable in time. However, in the case of PHA treatment it is difficult to assess the individual consistency because any repeated application of PHA causes an adaptive immune response to PHA (Tella et al. 2008) distinct by the involved immunological processes from 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.

Despite the individual consistency of the immune response to PHA is problematic to measure, we may assess it based on the stability of traits correlated with the response. Current evidence suggests that the magnitude of the skin-swelling induced by PHA may be reflected by colourful ornamental traits such as carotenoid-based bill- (McGraw & Ardia 2003; Faivre et al. 2003b); but see Biard et al. 2009) or skin-colouration (Perez-Rodriguez et al. 2008; Mougeot 2008; Bonato et al. 2009). Although these traits seem to show some individual consistency (Perez-Rodriguez 2008), it is also known that their colouration may undergo rapid changes after alternation of the health state (McGraw & Ardia 2003; Faivre et al. 2003a); but see Biard et al. 2009). 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 avian colourful ornaments are, however, feather-based and much less is currently known about their relationship to the PHA-triggered immune responsiveness. The linkage of the PHA-induced skin-swelling test results to the melanin-based plumage ornamentation was found by some studies (Gonzalez et al. 1999a; Jacquin et al. 2011) while no such a pattern was reported by others (Gonzalez et al. 1999b). Similar pattern was also described for the structurally based feather UV-colouration (Parn et al. 2005; Griggio et al. 2010). Nevertheless, melanin-based and in some species also structural-based ornaments reflect more social status of their bearer than its condition and health (McGraw et al. 2003; Badyaev & Young 2004). On the other hand, carotenoid-based ornaments are commonly assumed as valuable indicators of the health state and parasite resistance (see e.g. Vinkler & Albrecht 2010). Unfortunately, as the current evidence is rather limited and does not show any clear pattern (Navara & Hill 2003; Saks et al. 2003b), we presently lack much information on the predictive value of carotenoid-based feather ornamentation regarding the magnitude of the PHA-induced immune response indicating the pro-inflammatory potential of the individual. The same is also true for associations of other long-term condition-dependent traits such as ptilochronological traits to the outcome of the PHA skin-swelling test.

In the present study we focused on the possible linkage between magnitude of the PHA-triggered immune response six hours after PHA application and several traits associated with long-term condition in scarlet rosetfinch (*Carpodacus erythrinus*). Since the red breast plumage ornamental colouration in this species seems to be an important predictor of individual fitness (Albrecht et al. 2009), we decided to focus on this trait. In rosetfinch males captured before breeding we analysed three components of the ornamental colouration: hue, saturation and brightness. Furthermore, we assessed nutritional condition during the feather moult by measuring the mean growth bar width

(a ptilochronological marker of the feather growth speed; Grubb 2006) and recorded measures of morphometric indicators of condition such as size and size-standardised weight (body mass). To provide clarification to 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 characterising the cellular composition of the inflammation.

METHODS

Field Procedures

In 2006-2009 scarlet rosefinch males were trapped into standard ornithological mist-nets after their spring arrival to the breeding grounds (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 at the age of three years or older (considered as adult based on their plumage colouration) were caught and examined according to the following protocol. First, a standard measurement of individual weight was performed using Pesola spring balance (accuracy 0.5 g) and a second outermost rectrix from the left side of the tail was collected for the ptilochronological assay. Thereafter, the PHA treatment was performed. In this study we adopted the simplified protocol described by Smits et al. (1999) with the following modifications. The treatment dosage utilised in our study was 0.10 mg of PHA-P dissolved in 20 µl of DPBS (product numbers L8754 and D5652 respectively; Sigma-Aldrich, St Louis, MO, USA; see Vinkler et al. 2010a). After the injection each bird was placed individually into a cage (41x23x23cm) with access to food and water ad libitum, located at a calm shady place with no visual contact with the surroundings of the cage or with other birds. Cages were periodically controlled to ensure that all birds feed and do not show any signs of stress. The magnitude of the swelling reaction was measured after 6 ± 0.5 hours to avoid negative impact of caging on male natural mating (for the usage of 6h 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 6h and 24h period). From the histological point of view we investigated the early phase of the PHA-induced inflammation. Each metrical tissue-magnitude measurement was performed three times with accuracy to 0.01 mm and the average was taken for the 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 hours after the treatment minus the average thickness before the PHA injection. In 2009 a biopsy sample of the tissue was collected after measuring the swelling magnitude and fixed in 10% formalin for later histological analysis. Then, another measurement of the individual weight was performed and the tarsus length was recorded (accuracy 0.01 mm). The tarsus length served as a general estimate of individual's size and the weight was later divided by tarsus length to obtain a measurement of weight standardized on size (hereafter referred as mass). Food-intake during the caging had no effect on the magnitude of the PHA-induced swelling response as revealed by the non-significant association between the weight change (individual weight prior caging minus post caging) and the PHA-induced swelling response index ($P = 0.32$). In general, the birds did not

loose weight during the caging (paired t-test for the weight change, $P = 0.30$). Finally, a standardized digital image of the ventral ornamented side of the bird was taken with a digital camera (see below), the bird was ringed with a standard steel ring of the Czech ringing station (N MUSEUM PRAHA) and released. The research was approved by 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 Colouration

For the analysis of the ornamental colouration standard digital photographs were used. As the precise description of the method adopted in this study is given in detail elsewhere (Albrecht et al. 2009), we will mention it here only briefly. All photographs were taken with Olympus C-765 digital camera (Olympus Corp., Tokyo, Japan) under standard lighting conditions using an electronic flash. To enable even more precise standardisation of the image colours colour swatches (grey card GC 18 and colour & grey chart Q 14; Danes-Picta, Praha, Czech Republic) were placed into close proximity of the animal. Photographs were analysed using Adobe PHOTOSHOP CS.3 software, version 10.0 (Adobe Systems Inc., San Jose, California). First, all images were standardised according to the 50% grey and red colour on the swatch. Values of hue, saturation and brightness (HSB colour space) were then measured in 10 points (each of area 5x5 pixels) randomly distributed over the ornamented breast colour patch. For the following analysis only average values were taken. Hue (with higher values in more yellowish colouration) is in finches indicative of the type of carotenoids present in the feathers (Inouye et al. 2001); saturation was shown to reflect the quantity of the carotenoid content (Saks et al. 2003a) and brightness appears to be determined by the structure of the feather microsurface (Shawkey et al. 2007).

Ptilochronological Assay

To measure the nutritional differences among individual males in the time of moulting ptilochronological measurements of the feather growth bars were adopted (Grubb 2006). For each individual a standard digital image of the collected rectrix with 50 mm scale was taken with BenQ 5550T scanner (BenQ Corporation, Taipei, Taiwan). These digital images were then adjusted in Corel PHOTO-PAINT X3 software (Corel Corporation, Ottawa, Canada) by 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>). For estimation of the mean width of the growth bars a segment of 8 bars with the centre in 2/3 of the feather was used.

Histological Analysis

In 15 individuals captured in 2009 a tissue sample (2 mm in diameter) was punched from the centre of the inoculation site after the metric measurement of the swelling response. All these samples were immediately fixed with 10% buffered formalin and later used for histological examination of the

tissue. As the procedure was reported earlier in Vinkler et al. (2010a) in detail, we will describe it here only briefly. Samples were embedded into paraffin, sectioned and stained with hematoxylin and eosin (performed by BIOLAB Praha laboratory, Czech Republic). Three sections were made out of the middle part of each biopsy sample and in each of these sections three independent places were photographed (i.e. 9 photographs per sample) under 40x 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 mm x 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 mm² was obtained for following categories of cells: lymphocytes, monocytes, heterophils, basophils, erythrocytes and thrombocytes. Two individuals had to be excluded from the analysis due to a 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 analyze effects of hue, saturation and brightness of male feather colouration, body mass, individual size and mean growth bar width on the magnitude of PHA swelling reaction, general linear mixed-effects model was used with year (2006-2009) treated as a random effect. Prior to analysis, data were checked for normality using Kolmogorov-Smirnov test. Minimal adequate model (i.e. a model with all terms significant) was obtained by backward eliminations 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 MAM 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 performed on biopsy samples collected from 13 males captured in 2009 revealed the following cellular composition of the swollen-tissue infiltrate (Mean \pm SE cells per 0.0729 mm²): lymphocytes 81.54 \pm 11.98, macrophages 9.92 \pm 2.33, heterophils 46.92 \pm 9.55, basophils 184.62 \pm 20.34, erythrocytes 23.08 \pm 4.91, thrombocytes 108.92 \pm 9.34. Thus, the immune response to PHA was a typical inflammation with a dominant infiltration of basophils (39.91 % on average). We therefore analysed the relationship between tissue basophil count and magnitude of the swelling response. We have found a significant positive relationship between the metrical magnitude of the swelling and the number of basophils within the infiltrate explaining more than 30 % of the variability present within the data on patagium thickness change due to the swelling response to PHA (Regression: $y = 0.002x + 0.6436$, $R^2 = 0.309$, $F_{1,11} = 4.93$, $P = 0.048$; see Fig.1.).

The effect of long-term condition on the PHA-induced inflammatory response was analysed within the complete dataset ($N = 45$) with the random effect of year. Most condition-related parameters,

namely male ornament saturation ($P = 0.004$; Fig.2.), male ornament brightness ($P = 0.020$; Fig.3.), individual size ($P = 0.005$) and mean growth bar width ($P = 0.002$), had a significant influence on the patagium thickness change due to the swelling response to PHA. We have found no significant effect of body mass or male ornament hue on the magnitude of the response (for both $P > 0.10$). The resultant minimal adequate model is summarised in Table 1. No correlation between mean growth bar width and any parameter of the ornamental colour has been found (in all cases $P > 0.5$). There was, however, a significant negative correlation between the mean growth bar width and individual size (Pearson's product-moment correlation: $r_p = -0.33$, $t_{43} = -2.28$, $P = 0.028$).

DISCUSSION

In this study we have shown two important aspects of the PHA-induced inflammatory response in scarlet rosefinch. Firstly, the swelling response is (at least partly) dependent on basophil activity, as these represented the most numerous cell type within the inflamed tissue 6 hours after the PHA application and there was a positive relationship between the metrical magnitude of the swelling response and the quantity of basophils within the tissue. Secondly, the PHA-response magnitude mirrors the long-term quality of the individual as it is significantly influenced by individual size, ornament colouration and nutritional condition in the time of moulting. However, the negative relationship between ornament saturation and the magnitude of the response and the positive relationship between ornament brightness and the magnitude of the response indicate that low quality males respond stronger than high quality ones. Thus, interestingly, our data do not fit the common interpretation line for the results of the PHA skin-swelling test and show the opposite pattern.

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 has been confirmed also in the present study. Contrary to other studies reporting massive basophile infiltration only in later phases of the response, following the heterophil influx (McCorkle et al. 1980; Martin et al. 2006) we have found as early as 6 hours after the PHA treatment basophils being the predominant cell type in the tissue. This is, however, not surprising given that basophils form a major cell type even in the healthy scarlet rosefinch peripheral blood (Vinkler et al. 2010b). Basophils represent a leukocyte type with crucial involvement in acute inflammatory response (Campbell & Ellis 2007). Our data 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 magnitude of the PHA-induced swelling has to be, however, treated with some caution due to the relatively small size of the sample of males available.

In our study we have revealed that the individual size is positively associated with the magnitude of the swelling response to PHA in 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 a larger volume of skin prone to inflammation. Although several authors also reported the relationship between the wing-web thickness change 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 (Hausmann et al. 2005; Nolan et al. 2006; 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 PHA-induced immunoresponsiveness. Mean growth bar width is a ptilochronological marker of condition state during autumn moult (Grubb 2006). The positive association between 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, physical capabilities and higher survival probability (Soler et al. 1999; Gonzalez et al. 1999b; Mougeot et al. 2009). However, given the negative correlation of the mean growth bar width with the animal 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 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 stronger than less metabolically active birds. This relationship, nevertheless, deserves further investigation as the interpretation of the observed association is so far, lacking other clues, rather speculative.

In species related to scarlet rosefinch (such as house finch, *Carpodacus mexicanus*) it was shown that males with more elaborated ornaments cope better with infectious pathogen diseases (Hill & Farmer 2005). Carotenoid-based ornamental colouration is composed of three independent variables: ornament hue (indicating mostly the type of pigments deposited), ornament saturation (indicating amount of carotenoids deposited with higher values in healthier males) and ornament brightness (indicating surface structure with higher values indicative of e.g. degradation of the feathers by bacteria (Inouye et al. 2001; Hill 2002; Saks et al. 2003a; Shawkey et al. 2007)). Our data 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 positive relationship to feather brightness. It was shown in male blackbird (*Turdus merula*; Faivre et al. 2003b), zebra finch (*Taeniopygia guttata*; 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) and ostrich (*Struthio camelus*; Bonato et al. 2009) that beak, comb and skin carotenoid-based colouration is often in positive association with PHA responsiveness. In blue-footed booby (*Sula nebouxii*) Velando et al. (2006) found a positive relationship between the blue structural-and-carotenoid-based colouration and the intensity of the PHA-induced immune response. Similar results were also reported by Griggio et al. (2010) for the

UV reflectance of plumage in budgerigars (*Melopsittacus undulatus*) and by Nolan et al. (2006) for the pterin-based breast plumage colouration in adult king penguin (*Aptenodytes patagonicus*) males. Also in barn swallows (*Hirundo rustica*) and blue peafowl (*Pavo cristatus*) it was shown that the tail length (a sexually selected ornament) is positively associated with PHA response (Saino et al. 2002; Møller & Petrie 2002). On the contrary, several other studies did not find any clear association between the magnitude of the swelling response to PHA and sexually selected ornamentation either morphological (Buchholz et al. 2004), melanin-based (Gonzalez et al. 1999b; Parn et al. 2005) or UV-based (Nolan et al. 2006). The same is also true for another study focused on carotenoid-based bill colouration in blackbird males (Biard et al. 2009). Furthermore, Navara & Hill (2003) and Saks et al. (2003b) found no association between the carotenoid-pigmented plumage colouration and PHA responsiveness in American goldfinch (*Carduelis tristis*) and greenfinch (*Carduelis chloris*), respectively. Even a negative relationship was reported between the PHA response and components of male ornamentation (in peafowl and the diameter of the train ocelli, (Møller & Petrie 2002), and in common yellowthroat, *Geothlypis trichas*, the black mask size, (Garvin et al. 2008)). Gonzalez et al. (1999a) also showed that in house sparrow (*Passer domesticus*) the relationship between the magnitude of the PHA-induced response and melanin-based ornamentation may vary from positive to negative in the course of year. This might be due to changes in the immunological mechanism of the response. However, as carotenoid-based colouration is an individually consistent trait (Perez-Rodriguez 2008), we may expect that in the long-term view also the PHA responsiveness might be individually consistent.

We believe that the clue for the interpretation of this disagreement of results originating from studies in different species lies 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). A reasonable critique showing the imperfections in our actual understanding of the underlying immunological mechanisms (Saks et al. 2006; Kennedy & Nager 2006; Owen & Clayton 2007) together with the histological (McCorkle et al. 1980; Martin et al. 2006; Vinkler et al. 2010a) and experimental (Sarv & Hõrak 2009; Vinkler et al. 2010a) evidence, nevertheless, suggest that at the current state of our knowledge the appellation ‘T-cell-mediated immunocompetence’ is in the case of PHA-induced immune response problematic. On the contrary, it seems that any clear evidence showing major involvement of T cells in the primary inflammatory immune response to PHA is missing (for further discussion see Vinkler et al. 2010a). Within this shifted interpretation framework it is, nonetheless, much more difficult to assume easily higher responsiveness to PHA as a ‘good’ trait. The stronger response may result, for instance, from currently activated inflammatory processes elsewhere in the organism. As emphasised 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, in some cases greater magnitude of the response may be indication of either non-effective anti-parasite protection or immunopathology (Graham et al. 2005). This can be tracked also in the results of

immunoecological studies. For instance, in male blue peafowl Møller & Petrie (2002) found 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). Moreover, in several passerine species it was found that 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 current health state, perhaps as a result of elevated pre-treatment blood levels of cells involved in inflammation (e.g. basophils or heterophils) and plasma-mediated pro-inflammatory cytokines. 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. Our data, however, do not allow for the investigation of temporal dynamics of the response.

To conclude, although carotenoid-pigmented living tissues (i.e. dynamic traits such as e.g. beak rhamphoteca 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. In scarlet rosefinch we have found a negative association between the magnitude of the PHA-induced skin swelling response and the feather-based ornamental colouration suggesting that inferior individuals respond stronger than high quality ones. This result is meaningful given the known immunological nature of the response in this species. As basophils seem to play a crucial role in the inflammation in scarlet rosefinch, further research should be focused on deeper investigation of immunological activity of basophils in this species.

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Figures

Fig.1. Association between the tissue basophile count and the magnitude of swelling response in scarlet rosefinch males after subcutaneous PHA application ($F = 4.93$, $N = 13$, $P = 0.048$).

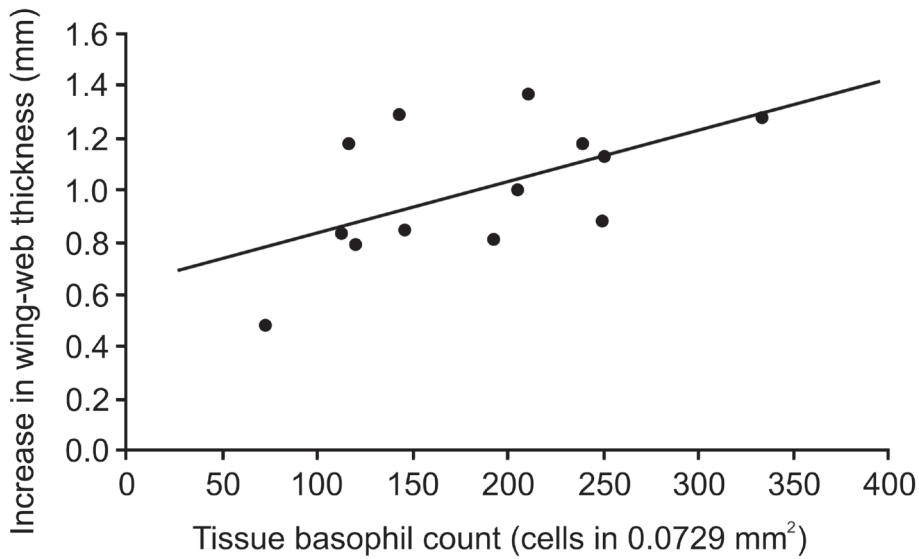


Fig.2. Association between the ornament saturation and the magnitude of swelling response after subcutaneous PHA application ($t = -2.77$; $N = 45$; $P = 0.004$).

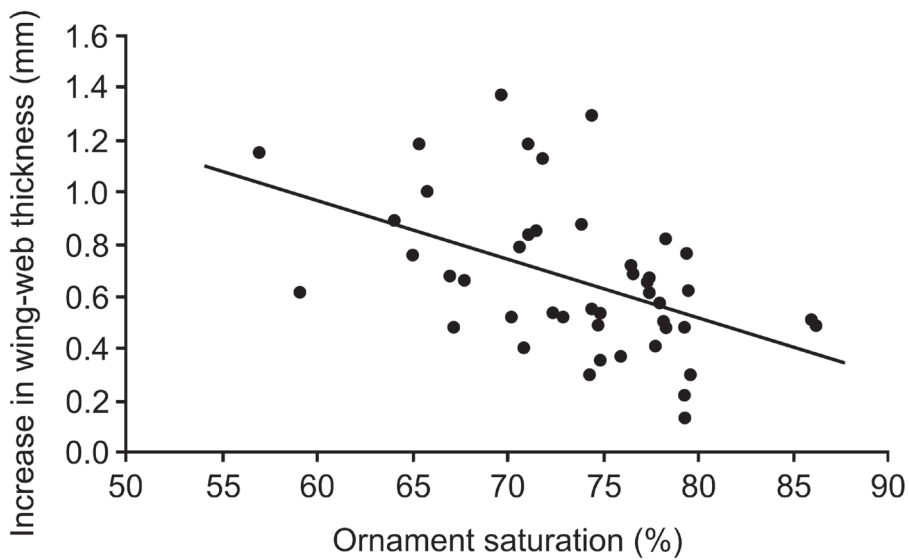
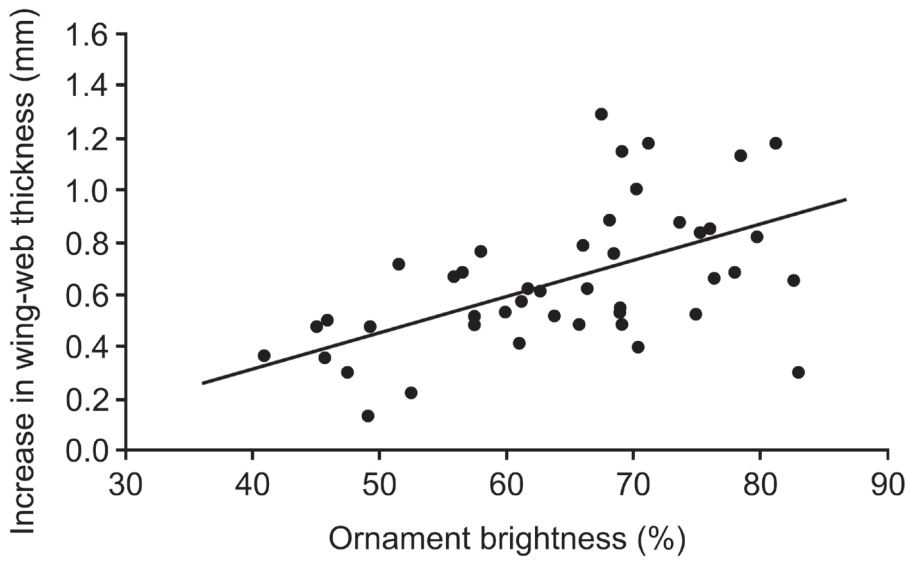


Fig.3. Association between the ornament brightness and the magnitude of swelling response after subcutaneous PHA application ($t = 2.24$; $N = 45$; $P = 0.020$).



Tables

Table 1: Minimal adequate model for the condition-dependency of magnitude of the PHA-induced immune response in scarlet rosefinch males, $\chi^2_4 = 21.00$, $N = 45$, $P < 0.001$. Analysis is based on mixed effect general linear model with year (2006-2009) treated as a random effect.

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