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ULVI KARU

Antioxidant protection, carotenoids and coccidians in greenfinches – assessment of the costs of immune activation and mechanisms of parasite resistance in a passerine with carotenoid-based ornaments

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I Hõrak, P., Saks, L**.**, Karu, U., Ots, I., Surai, P.F. & McGraw, K.J. (2004) How coccidian parasites affect health and appearance of greenfinches. *Journal of Animal Ecology* 73: 935–947.

II Hõrak, P., Saks, L., Karu, U. & Ots, I. (2006) Host resistance and parasite virulence in greenfinch coccidiosis. *Journal of Evolutionary Biology* 19: 277–288.

III Saks L., Karu U., Ots, I., Hõrak, P. (2006) Do standard measures of immunocompetence reflect parasite resistance? The case of Greenfinch coccidiosis. *Functional Ecology* 20: 75–82.

IV Hõrak, P., Zilmer, M., Saks, L., Ots, I., Karu, U. & Zilmer, K. (2006) Antioxidant protection, carotenoids, and the costs of immune challenge in greenfinches. *Journal of Experimental Biology* 209: 4329–4338.

V Hõrak, P., Saks, L, Zilmer, M., Karu, U. & Zilmer, K. (2007) Do dietary antioxidants alleviate the cost of immune activation? An experiment with greenfinches. *American Naturalist* 170: 625–635.

VI Karu, U., Saks, L., Hõrak, P. (2008) Plumage coloration is not affected by vitamin E supplementation in male greenfinches. *Ecological Research,* in press.

Author's contribution to the papers:

	I	**II**	**III**	**IV**	**V**	**VI**
Original idea						*
Study design	*	*	*	*	*	*
Data collection	*	*	*	*	*	*
Data analysis	*	*	*	*		*
Manuscript preparation	*	*	*			*

1. INTRODUCTION

Selection pressure, excreted by parasites and pathogens is currently recognised as one of the most important evolutionary forces (reviewed in Owens & Wilson 1999; Zuk & Stoehr 2002; Graham *et al.* 2005; Medley 2007). This coevolutionary process has led hosts to develop defence mechanisms, consisting primarily of the immune system, which (especially among vertebrates) has reached extreme complexity and tight integration with other physiological functions of an organism. The intuitive understanding that such a sophisticated defence system is not cost-free has promoted an increasing interest of evolutionary animal ecologists in the potential role of immune defence in shaping of life-histories and sexually selected traits. This interest has given rise to a discipline labelled as ecological immunology (or immunoecology) by Sheldon & Verhulst in 1996. The central paradigm of immunoecology is that immune function interacts with the general health state of an organism and competes for the resources that can be allocated to other activities, such as maintenance, reproduction, and development of the ornamental traits. Hence, one may expect trade-offs in resource allocation between immune function and other vital needs of an organism.

Previous studies on different animal species have demonstrated that activation of the immune system can indeed suppress reproduction, either by reduction of the quality of sexual signals or parental care. Additionally, some evidence about immunosuppression due to increased reproductive investments has been found (reviewed by Zuk & Stoehr 2002). However, the question about the currencies used for paying the costs of activation of immune defences has remained poorly understood for the animal ecologists. The traditional view of animal ecologists, namely that the costs involved in life-history trade-offs are basically energetic, has been challenged by claims that energetic demands required for maintenance of immune function and for mounting specific immune responses are negligible (Klasing & Leshchinsky 1999; Klasing 2004). Furthermore, experimental tests of these ideas have given contradictory results (e.g., Ots *et al.* 2001 vs. Hõrak *et al.* 2003). Therefore, an alternative hypothesis, proposing that costs of immune responses are primarily caused by the accompanying immunopathological damages, is becoming increasingly popular (von Schantz *et al.* 1999; Råberg *et al.* 1998). Oxidative stress (OS), caused by production of oxidative metabolites and free radicals during immune responses is currently considered as one of the most serious sources of immunopathological damages (e.g., von Schantz *et al.* 1999; Hensley *et al.* 2000). Oxidative products and free radicals, which are highly reactive by-products of normal metabolism and immune defence, can cause extensive oxidative damage to biomoleculs such as nucleic acids, proteins and lipids if an organism lacks sufficient antioxidant protection. Processes like neurogenesis, immunomodulation and expression of MHC molecules are particularly susceptible to OS. Additionally, OS is believed to play a major role in several processes leading to senescence (Beckman & Ames 1998).

Antioxidants (AO) are chemical substances that scavenge (deactivate) free radicals by donating them missing electrons. Exogenous AO (such as fat-soluble vitamins E and A, carotenoids) must be obtained by food while endogenous AO (cf. enzymes like catalase, superoxide dismutase, glutathione peroxidase, and uric acid; in some avian species also ascorbic acid) are synthesized by organism. Due to medical importance, the previous research on OS on vertebrates has been mainly concentrated on mammals (with the few exceptions such as Alonso-Alvarez *et al.* 2004 and Wiersma *et al.* 2004, published in the time of accomplishing first papers included in this thesis). Traditional research objects of animal ecologists – passerine birds – differ from mammals by their higher metabolic rates (2–2.5 × of that of mammals), which should result on higher exposure to OS. Without unique protective mechanisms against the potential for oxidative damage, birds would be short lived and age more rapidly than mammals. Yet the lifespans of birds are longer than those of similar-sized mammals, which suggests that they have evolved somewhat different mechanisms to limit the damage caused by OS (e.g., Klandorf *et al.* 2001). Indeed, avian species have higher levels of antioxidants (carotenoids, tocopherols, uric acid) compared to mammals which is not probably co-incidental to the pattern that avian signal traits (sexually selected ornaments, warning colours, parent-offspring visual cues) are disproportionally often based on carotenoid pigments (Møller *et al.* 2000).

Carotenoid-based signals have been in the scope of extensive research by animal ecologists since understanding of their possible dual role in health maintenance and communication (reviewed by Olson & Owens 1998; McGraw & Ardia 2003). Currently, it is widely accepted that carotenoid-based visual characters enable individuals to signal their phenotypic and genetic quality: if an individual has only a limited amount of carotenoids at its disposal, then it can invest them efficiently into signal traits only when it does not need carotenoids for maintenance purpose at the same time. In such way, carotenoid-based traits may either signal foraging (and food absorption) efficiency, immunocompetence or antioxidative potential of signallers. The question of the relative importance of these aspects is currently under lively debate (Hill 1999; McGraw & Hill 2000a; Møller *et al.* 2000; Lozano 2001; Costantini *et al.* 2007; Isaksson *et al.* 2007; Isaksson & Andersson 2008; Costantini & Møller 2008). Interestingly, the primary importance of carotenoids as antioxidants has been recently debated by Hartley & Kennedy (2004) who proposed an alternative hypothesis about their biological role: namely, if carotenoids are signalling the excellence of antioxidant resources, then it is as indicators of those resources and not as an advertisement for the carotenoids themselves. If another, non-pigmented AO (such as AO enzymes, and vitamins E and C) are more important biological protectants against free-radical mediated OS than are carotenoids and their metabolites, then organisms just can to rely on carotenoids to advertise their health state because the presence of other AO would mitigate strongly against the oxidative decolouration of carotenoids. This hypothesis represents

an important challenge to the currently prevailing hypotheses of biological function of carotenoids and also stresses the importance of understanding the biochemical physiological processes underlying the expression of signal traits. When I began to work with this thesis, no experimental tests had been performed on this hypothesis.

One of the main aims of this thesis is elucidation of relative importance of carotenoids and endogenous antioxidants in the context of expression of signal traits and immune function in greenfinches – passerines with sexually selected carotenoid-based plumage ornaments. Because endogenous and exogenous AO comprise an integrated regulatory system, the importance of different components of this system can be inferred by manipulation of one or more of its key elements. By such manipulations of immune function and dietary antioxidant availability, I expected to establish the factors that appear either critical, limiting, or unimportant for antioxidant protection in birds under various conditions. These issues are addressed in papers **IV, V**, and **VI** of this thesis. However, artificial, non-pathogenic immune challenges used as a proxy of „immunocompetence" by animal ecologists may not necessarily capture the functional aspects of immune defences that are used to fight off real pathogens (Owens & Wilson 1999; Adamo 2004; Owens & Clayton 2007). I therefore chose to study the problems of health impact of antioxidant protection and oxidative stress in a model where I could experimentally manipulate intensity of parasitic infection, namely the intestinal coccidiosis. Furthermore, to understand the physiological impacts of immune activation and antioxidant supplementation, I needed to account and control for the physiological impact of parasites in my study system. The first half of my thesis therefore deals with issues of coccidiosis – how do these parasites affect the health and appearance of their hosts (**I**), what factors determine host resistance and parasite virulence (**II**) and how is the parasite resistance related to standard measures of immunocompetence, assessed on the responsiveness to artificial antigens (**III**).

Avian coccidiosis seems to be a potentially promising model for such experiments. Coccidia from the genus *Isospora* (Protozoa, Apicomplexa, Sporozoa, Coccidia) are widely distributed (reviewed e.g., in Giacomo *et al.* 1997; McGraw & Hill 2000b; Duszynsky *et al.* 2004) and can cause severe disease, even death among passerine birds (Box 1977; Sironi 1994; Giacomo *et al.* 1997). Moreover, it is known from the studies on domestic chicken that coccidians from the genus *Eimeria* inhibit the absorption of several essential dietary components, including carotenoids in the intestine (e.g., Allen 1987; Allen 1997; Allen & Fetterer 2002) and can cause depression of carotenoid-based pigmentation (Tyczkowsky *et al.* 1991). How the physiology and ornament expression of greenfinches is affected by the infection with intestinal coccidian parasite, *Isospora lacazei*, is studied in paper **I**. The existence of concurrent polymorphism among the host resistance and parasite virulence is one of the main assumptions of the models of host-parasite coevolution (reviewed e.g., in Clayton & Moore 1997; Little 2002; Summers *et al.* 2003).

However, this important assumption has not been tested in wild bird-parasite model systems. The issue of variation in the host resistance and parasite virulence is addressed in paper **II**, while the paper **III** aims at understanding the utility of two common immune assays – phytohemagglutinin skin test and antibody production against foreign erythrocytes for assessment of real parasite resistance in the model of coccidiosis. Aforementioned immunological assays are extensively used in immunoecological and immunotoxicological research (reviewed in Schrank *et al.* 1990; Smits *et al.* 1999; Martin *et al.* 2006) where they are believed to estimate the general potential of the immune system to respond to the novel challenges (e.g., Faivre *et al.* 2003; Smits & Baos 2004; Møller & Saino 2004). The implicit assumption of such tests, namely that immunoresponsiveness correlates positively with the ability to resist diseases, however, has remained rather untested, at least in the context of avian immunoecological research.

2. STUDY SPECIES

The Greenfinch

Greenfinches are medium-sized (ca. 28 g), sexually dichromatic gregarious seed-eating passerines native to the western Palearctic region. Males are larger and more colourful than females, with old males developing olive-green plumage on the back, bright or greenish yellow colour on the breast, and striking bright yellow markings on the primaries, primary coverts and sides of the tail feathers. Females are more olive-brown and yellowish-buff, having faint brown streaks on back and lacking full yellow tints in their plumage (Cramp & Perrins 1994). The yellow colour of these feathers is based on canary xanthophylls A and B and their *cis* isomers (Saks *et al.* 2003), which are produced by interconversion of dietary lutein and zeaxanthin (Surai 2002). Intensity of yellow coloration of male greenfinches reflects the amount of carotenoids deposited into feathers (Saks *et al.* 2003). Carotenoid-based plumage coloration of male greenfinches is sexually selected (Eley 1991) and is sensitive to viral (Lindström and Lundström 2000) and hematozoan (Merilä *et al.* 1999) infections.

Coccidia

Coccidia of the genus Isospora are obligate intracellular parasitic protozoa infecting a wide range of songbirds species in the wild (rewieved by Giacomo *et al.* 1997; McGraw & Hill 2000b). A host becomes infected when it ingests oocysts that have been passed in the faeces of another host. The oocyst excysts in the epithelial cells of intestinal mucosa and liberates sporozoites from its contents. The sporozoites penetrate the cells of the host's small intestine and reproduce asexually. In case of passerine birds, the first-generation sporozoites may also invade liver, spleen and lungs (atoxoplasmosis, e.g., Giacomo *et al.* 1997). In the epithelial cells of intestine, each generation of asexual reproduction produces multiple merozoites that infect new cells. This stage of the infection can result in destruction of massive numbers of cells in the host's small intestine and, ultimately, lead to the host's death (e.g., Box 1977; Sironi 1994). Some of the merozoites that enter the host's cells transform into gametocytes. The gametocytes transform into gametes, the gametes fuse, and the resulting zygote begins to develop into an oocyst. The developing oocyst escapes from the host's cell, and it is passed in the host's faeces. The destruction of epithelial cells of small intestine during the reproduction of parasites is probably the main pathological effect of the Isosporan infections, which can cause a drastic reduction in digestive and absorptive capacity of mucosa (e.g., Ruff & Fuller 1975; Hoste 2001). The pathogeneicity of Isosporan

coccidiosis is well documented (Box 1977; Sironi 1994; Giacomo *et al.* 1997; **I**) and therefore, it is likely that these parasites can be an important evolutionary force for passerine birds.

3. RESULTS AND DISCUSSION

3.1. Effect of coccidian parasites on health and appearance of greenfinches (I)

The concept of parasite-mediated sexual selection proposes that parasite invasion may decrease the expression of secondary sexual characters while resources for sexual advertisement are needed to fight off the parasites (Hamilton & Zuk 1982). It has been proposed that expression of different types of ornamental traits is suppressed by oxidative stress, generated in the process of extensive production of free radicals during immune response (von Schantz *et al.* 1999). Bright carotenoid-based integumental colours have been considered particularly sensitive to oxidative stress and host infection status (Lozano 1994). The aim of this study was to examine the mechanisms by which parasites can affect the expression of ornamental traits in captive greenfinch. The natural infections of intestinal coccidia *Isospora lacazei* were suppressed and half the birds were experimentally infected with a mixture of different Isosporan strains while another half continued receiving medication. Eighty-nine per cent of birds hosted chronic Isosporan infection before the experiment. However, experimental inoculation resulted in 263-fold difference in oocyst output between medicated and infected birds and drastic decrease in body mass, serum carotenoid, vitamin E, triglyceride and albumin concentration.

Isosporan parasites of cardueline finches have been shown to damage most extensively the duodenal and jejunal part of the intestine, destructing epithelial cells and causing malabsorption (Giacomo *et al.* 1997). In domestic chicken, these intestinal parts have shown to be responsible for absorption of proteins (jejunum) and fats (duodenum) (Turk 1974) as well as vitamin E and carotenoids (Surai 2002). In humans and domestic chickens, lipid malabsorption causes decrease in plasma carotenoids and is a common cause of decreased plasma vitamin E (reviewed in Allen & Fetterer 2002). Moreover, decreased triglyceride and albumin concentrations also are indicative to malnutrition. These infection-induced effects upon host nutritional status were evidently severe enough to lead to significantly lower body mass in the infected compared to the medicated group during the peak infection phase. Loss of body mass due to coccidiosis has been shown in many studies on chickens (reviewed in Allen *et al.* 1998) but also on free-living birds (Cooper *et al.* 1989, McGraw & Hill 2000b; Costa & Macedo 2005).

Additionally, infected birds had higher concentration of heterophils in blood compared to the medicated ones. The increase of peripheral heterophil count in infected birds may be associated with increased traffic of these phagocytozing cells to the intestinal mucosa, where they participate in the removal of debris (Rose *et al.* 1979). Such phagocytotic processes are often linked to remarkable tissue damage caused by the production of proteolytic enzymes and free

radicals. This could be additional reason for the drop of serum carotenoids and vitamin E during the peak infection phase as these substances might have been depleted as scavenges of free radicals produced during the immune response (e.g., Allen 1997). Another reason for the reduction in blood carotenoid levels due to infection may be related to depressed synthesis of high density lipoproteins (the main carriers of carotenoids and vitamin E) in the intestinal mucosa, as has been shown during *Eimeria acervulina* infections in chickens (Allen 1987).

Experimentally infected birds also had less carotenoids in their tail and breast feathers, which resulted in greater reduction in the coloration of these feathers compared to the medicated birds. The depression of carotenoid-based ornaments by coccidian parasites has been demonstrated also in two other studies on cardueline finches (Brawner *et al.* 2000; McGraw & Hill 2000b). Thus, the bright carotenoid-based ornamental plumage in male greenfinches indicates their better nutritional status and may be an indicator of the ability to resist the currently prevailing Isosporan parasite. This would be consistent with the concept of parasite-mediated sexual selection which states that only individuals most resistant to infections can express elaborate ornamentation (Hamilton & Zuk 1982).

Interestingly, with regard to sexual selection and mate preferences, a recent study on blue-black grassquits (*Volatinia jacarina*; Aguilar *et al.* 2008) showed that although the coccidian parasite levels correlated negatively with body condition and expression of behavioural displays in males, females did not prefer nonparasitized males. Thus, more studies are needed to assess the link between male disease status and female preferences in the context of parasite-mediated sexual selection.

3.2. Host resistance and parasite virulence (II)

The question why different host individuals within a population differ with respect to infection resistance is of fundamental importance for understanding the mechanisms of parasite-mediated selection, PMSS (Hamilton & Zuk 1982). A crucial assumption of most hypotheses of PMSS is that the hosts should vary either genetically or phenotypically in resistance to infections while parasites should vary in virulence. These issues of variation in host resistance and parasite virulence were assessed in another coccidian infection experiment. In order to test whether different Isosporan strains vary in virulence and whether different host individuals vary in resistance to infection, greenfinches were infected with Isosporan parasites, originating either from single or multiple hosts. Birds were allocated to four treatment groups – the first group („own“) was inoculated with the oocysts collected from their own faeces, the second („mixture“) got mixture of oocysts collected from six heavily infected birds, the third („single strain“) received oocysts collected from a single bird. The fourth

group consisted of birds, who had naturally low infection intensities („initially low") got the same mixture of oocysts as the „mixture" group.

Infection with multiple strains resulted in greater virulence than single-strain infection, suggesting that parasites originating from different host individuals are genetically diverse. Considering that parasite strains differ in their virulence, hosts are more likely to encounter virulent parasites from multiple rather than from single infections. With regard to developing protective immunity against subsequent infection with the same strains, average infection intensity did not decrease after secondary infection among birds that were repeatedly infected with the same heterologous parasite. Therefore, exposure to novel strains did not result in protective immunity against the subsequent infection with the same strains. Thus, this result suggests that individuals really differed in their general capability to resist coccidiosis. For instance, a recent study of *E. maxima* infection in chickens (Smith *et al.* 2002) has demonstrated full protective immunity against the reinfection with the same strain of the parasite, while cross-protection against heterologous parasite strain varied from zero to almost 100%, depending on host genetics. Yet it is at present unknown whether genetic variation in parasite resistance is also responsible for the differential susceptibility to coccidian infection in wild birds.

However, the result that reinfection of host with their own parasite fauna resulted in lower infection intensities than infection with multiple strains, indicates that hosts can tolerate their own, previously acquired parasites better than novel ones. Surprisingly, birds infected with a single novel strain had infection dynamics virtually indistinguishable from the birds infected with their own parasites. Given that coccidians invading previously infected host are expected to face fierce competition by preceding strains (e.g., Williams 1998) and more virulent parasite strains are generally supposed to outcompete milder strains (reviewed in Wedekind 1999), it seems plausible that coccidians from a single host were less likely to compete with pre-existing strains than coccidians originating from multiple hosts.

Birds from "initially low" group retained low infection intensity throughout the experiment, although they received exactly the same heterologous inoculum as the birds with average pre-experimental infection intensity. This result implies that natural infection intensities also confer information about the ability of individuals to resist novel strains. Thus, this finding contributes important information in the context of immunoecological research and is appropriate for the models of parasite-mediated selection, which assume intra-population variation in host resistance (Clayton 1991; McLennan & Brooks 1991; Poulin & Vickery 1993).

Additionally, this study provides support for assumption that host resistance varies proportionally with parasite virulence (damage caused to the host) and parasite fitness (its reproductive rate). This was indicated by the patterns in plasma triglyceride levels and body mass dynamics in different treatment groups, which were generally inversely proportional to the patterns of oocyst

output in the same time periods. Consequently, the experimental inoculation caused significant changes in physiology of treated birds.

Altogether, these results suggest that the outcome of coccidian infection in greenfinches may depend on concurrent variation in host resistance, parasite virulence and their interaction.

3.3. How reliable are standard measures of immunocompetence? (III)

Parasite-mediated selection has been in the scope of extensive research by animal ecologists (Clayton & Moore 1997; Zuk & Stoehr 2002). Such studies require estimation of disease resistance. However, the estimation of individuals' parasite resistance in the field is often complicated. Because of difficulties in estimation of actual parasite resistance, researchers often rely on surrogate measures of immunocompetence, assuming that these measures reflect individuals` ability to resist disease or at least, indicate the relative "strength" of the immune system (e.g., Faivre *et al.* 2003; Smits & Baos 2005; Møller & Saino 2004). However, the assumption that immunoresponsiveness always correlates with the disease resistance has been questioned (Owens & Wilson 1999; Adamo 2004, Roulin *et al.* 2007). The questions of whether and how the measurements of immunocompetence obtained from standard immune challenge tests reflect real parasite resistance was the topic of the third paper of this thesis.

In order to test whether individuals' ability to mount immune responses against artificial antigens reflects their resistance to coccidiosis, the birds were injected with phytohemagglutinin to measure the general potential for cell-mediated immune response and sheep red blood cells (SRBC) to measure the ability for antibody production. These two assays are often used as the estimates of immunocompetence in the studies of wild birds (Smits & Baos 2005), which, in turn, is believed to be ultimately linked to fitness via general parasite resistance (Gustafsson *et al.* 1994; Møller *et al.* 1999). Additionally, to test whether immune responsiveness correlates with individuals' nutritional status, correlations between plasma triglyceride concentrations, body mass and magnitude of immune responses were measured.

Experimental infection with coccidians from single vs. mixed strains (see **II**) did not affect immune response to novel antigens. On the basis of birds ability to resist novel parasite strains they were divided into "vulnerables" – whose infection intensity increased after the experimental infection – and "non-vulnerables" – whose infection intensity decreased. Among non-vulnerables, infection intensities correlated negatively and condition indices correlated positively with SRBC antibody titre. However, no such patterns emerged among vulnerable birds. Moreover, among non-vulnerables, SRBC titres also clearly reflected their nutritional state, correlating positively with body mass

and plasma triglyceride levels. Coccidian infections are known to cause decline in individual nutritional status (**I, II**) by damaging intestinal absorptive mucosa (e.g., Ruff and Fuller 1975; Hoste 2001). It thus seems possible that those non-susceptible birds who had less damaged intestine (were less inflicted by or more resistant to coccidian infection) were in better nutritional condition and thus, capable to rise a stronger humoral immune response against a novel antigen. On the other hand, it would be possible that postnatal selection for parasite resistance and/or immunoresponsiveness had eliminated the most susceptible individuals from the sample available for this experiment. This, in turn, might have obscured the expected positive relationship between parasite resistance and immunocompetence in this study.

Interestingly, birds that responded to new infection with an increase in infection intensities mounted stronger swelling response to PHA injection. Thus, if to assume that the magnitude of swelling response to PHA indeed reflects the animal's general potential for cell-mediated immunity (e.g., Smits *et al.* 1999; Martin *et al.* 2001), then it would appear that most immunocompetent individuals are least resistant to a real infections. However, it is important to note that immune response against coccidiosis is mainly based on activation of various cell populations including T-lymphocytes, NK cells and macrophages (Lillehoj & Trout 1996). During this process, cytokines are produced, which are also responsible for the cutaneous hypersensitivity reactions, induced by the PHA injection (e.g., Lillehoj 1998). It is therefore likely that birds that were not resistant to the experimental infection were actively fighting against it and had therefore up-regulated their T-cell mediated immune function. Similar enhancing effect of parasites on T-cell mediated immune function has been shown for house martins (*Delichion urbica)* experimentally infected with haematophagous bug *Oeciacus hirundinis* (Christe *et al.* 2000). Similarly, Gwinner *et al* (2000) found higher PHA responses in nestling starlings (*Sturnus vulgaris*) from nests with high ectoparasite loads, while experimental infection of chickens with a bacterium *Salmonella typhimurium* resulted in higher lymphocyte proliferative responses to mitogens and increased cytotoxic activity of NK cells (Lessard *et al.* 1995). Thus, assessing immunocompetence only with a single immune assay can be misleading. Individuals may be selected to invest differentially in various components of the immune system (Adamo 2004) and immune system may also face trade-offs between different arms of defence (Roulin *et al.* 2007). For instance, Forsman *et al* (2008) found that in nestling house wrens (*Troglodytes aedon*) humoral immune response was negatively related to cutaneous immune activity and positively related to plasma bactericidal activity among broods. These examples call for caution when interpreting the results of standard immune tests in the context of parasite resistance. Altogether, these results exemplify the diversity of outcomes when hosts immune system is simultaneously challenged by natural enemies and artificial antigens.

3.4. Antioxidant protection, carotenoids and the costs of immune challenge (IV)

Costs accompanying immune challenges are believed to play an important role in life-history trade-offs (e.g., Ilmonen *et al.* 2000; Mallon *et al.* 2003) and warranting the honesty of signal traits (e.g., Kilpimaa *et al.* 2004). However, the question as to what exactly makes the activation of immune defences costly has remained less clearly understood (e.g., Moret & Schmid-Hempel 2000; Schmid-Hempel & Ebert 2002; Zuk & Stoehr 2002). The traditional view of ecologists is that the costs involved in life-history trade-offs are basically energetic (reviewed by Råberg *et al.* 2002; Eraud *et al.* 2005). An alternative hypothesis proposes that costs of immune responses are primary caused by the accompanying immunopathological damages (von Schantz *et al.* 1999; Råberg *et al.* 1998), which may result from excess production of reactive compounds during immune responses (Halliwell & Gutteridge 2007). To control and neutralize free radicals, animals maintain a system of defences based on endo- and exogenous antioxidants. Of all the antioxidants, animal ecologist have paid a disproportionate amount of attention to carotenoids (Lozano 1994; Olson & Owens 1998; von Schantz *et al.* 1999; Møller *et al.* 2000). Carotenoid-based traits might either signal foraging efficiency, immunocompetence or antioxidative potential of signallers, however, the relative importance of these factors is currently under lively debate (Hill 1999; Lozano 2001; Hartley & Kennedy 2004; Costantini & Moller 2008). Our previous study demonstrated that brighter carotenoid-based plumage reflects infection-free status in greenfinch coccidiosis model (**I**). However, the antioxidant properties of carotenoids have remained poorly understood (El-Agamey *et al.* 2004; Halliwell and Gutteridge 2007; Costantini & Moller 2008).

In order to investigate the role of carotenoids as potential antioxidants and immunomodulators, half the birds received carotenoid supplementation, and the 2×2 factorial experiment was performed where half the birds in control and carotenoid supplemented groups were immune challenged with SRBC. The parameters of individual condition including intensity of coccidian infection, estimates of total antioxidant protection, plasma carotenoids and ability to mount a cell-mediated immune response were measured.

Measures of total antioxidativity did not correlate with plasma carotenoid levels. This result is inconsistent with Alonso-Alvares *et al* (2004) who showed that plasma carotenoid levels in captive zebra finches (*Taeniopygia guttata*) correlated positively with antioxidative protection (Alonso-Alvarez *et al.* 2004). However, several recent studies failed to detect a correlation between plasma carotenoids and indices of total antioxidativity (reviewed by Costantini *et al* 2007; Costantini & Moller 2008). One possible explanation for these results would be that local actions of carotenoids in specific tissues are not reflected at the systemic level, so that plasma antioxidant capacity is not affected. Another explanation would be that TAS and AOP assays, used for assessment of plasma

antioxidativity are incapable to reflect the action of lipid-soluble antioxidants (Yeum *et al.* 2004; Beretta *et al.* 2006). Finally, we cannot totally exclude explanation that systemic antioxidant properties of carotenoids in birds (except well-established protective effects on embryos and hatchings) might not appear as important as previously thought (Hartley & Kennedy 2004; Halliwell and Gutteridge 2007). However, carotenoids could exert beneficial effects by other mechanisms (Halliwell and Gutteridge 2007).

Humoral immune challenge suppressed the cell-mediated response to phytohemagglutinin, suggesting a trade-off between the uses of different arms of the immune system. This trade-off might be rooted in the basis of cross-regulation between humoral and cell-mediated immune responses (Mosmann & Coffmann 1989), in which humoral (Th2) immune responses exert anti-inflammatory action by negatively regulating Th1-cell-mediated immunity and *vice versa*. Although such cross-regulation have been frequently observed in mammal models (reviewed by Kidd 2003) and in chickens (Degen *et al.* 2005), the discovery of a suppressed cutaneous swelling response in response to humoral immune system activation in greenfinches is, to our knowledge, the first such evidence in wild birds. Recent study in nestling house wrens (*Troglodytes aedon*; Forsman *et al.* 2008) demonstrated that among broods, the magnitude of humoral immune response was negatively related to cutaneous immune activity and positively related to plasma bactericidal activity.

Immune challenge also reduced body-mass gain, but only among the carotenoid-depleted birds. The findings of reduced body mass, mass gain or growth in response to non-pathological immune challenge have been documented in several avian studies (e.g., Klasing *et al.* 1987; Fair *et al.* 1999; Ots *et al.* 2001; Bonneaud *et al.* 2003). Possible mechanisms include energy reallocation from maintenance to immune function (reviewed in Lochmiller & Deerenberg 2000; Demas & Sakaria 2005) or inflammation-induced sickness syndrome, which results in reduced food intake and locomotory activity (e.g., Bonneaud *et al.* 2003; Klasing 2004). In this context, our result about the lack of effect of immune challenge on body mass dynamics among carotenoid-supplemented birds indicate that certain somatic costs associated with immune system activation can be alleviated by carotenoids. However, no evidence for oxidative stress-induced immunopathological damages could be found because immune activation did not affect biomarkers of antioxidant protection or carotenoid levels. Therefore, alterations of the total plasma antioxidant potential do not play any important role in forming the costs of SRBC-induced immune activation. This result is inconsistent with Alonso-Alvarez *et al.* (2004), who showed that immune challenge with bacterial polysaccharide (LPS) significantly depressed plasma carotenoid levels in captive zebra finches. On the other hand, that study also failed in detection of the effect of LPS injection on whole blood antioxidant protection (Alonso-Alvarez *et al.* 2004).

Nevertheless, carotenoid supplementation inclined birds to fattening, indicating that lutein interfered with lipid metabolism. To our knowledge, such a

phenomenon has not been previously described in an abundant carotenoid literature. We can exclude the possibility that carotenoid supplementation might have alleviated the coccidian-induced intestinal damage, known to suppress plasma triglyceride levels (**I, II**) because none of our treatments interfered with the dynamics of infection. Although these results support the hypotheses of biological importance of carotenoids, they also exemplify the overwhelming complexity of their integrated ecophysiological functions.

3.5. The role of endo- and exogenous antioxidants in compensating the costs of immune activation (V)

As discussed previously (**IV**), to counteract harmful effects of reactive oxygen and nitrogen species (RONS), organisms rely on a complex antioxidant network that includes endogenous and exogenous antioxidants. An imbalance between the production of RONS and antioxidant defences cause oxidative stress, which is considered as one of the most serious sources of immunopathological damages (von Schantz *et al.* 1999, Splettstoesser & Schuff-Werner 2002; Vajdovich 2008). The aim of this study was to ascertain whether activation of the immune system exacts oxidative costs and to examine to what extent exogenous dietary antioxidants mitigate potential damage from RONS. For this purpose, greenfinches were supplemented with combinations of lutein and vitamin E, two exogenous antioxidants, and immune challenged with PHA.

Compared to controls, immune-challenged birds circulated more lipid peroxidation products but also increased total plasma antioxidativity. PHA injection in birds is known to activate complicated immunological cascade including effector cells and phagocytes such as basophils, heterophils and macrophages (Martin *et al.* 2006; Hõrak *et al.* 2000). Heterophils involved in this process represent the first line of immune defence in terms of ingestion and destruction of potential pathogens. During particle ingestion, they produce large amounts of RONS, which can damage also the phagocytes themselves and other exposed cells (Splettstoesser & Schuff-Werner 2002). Therefore, such inflammation-induced damage is most parsimonious explanation for the PHA-induced lipid peroxidation and would be consistent with evidence of occurrence of lipid peroxidation in inflammations associated with infectious and degenerative diseases (Romero *et al.* 1998). On the other hand, the result that immune-challenged birds also increased total plasma antioxidativity is consistent with those observed in exercise-induced inflammation, where plasma antioxidativity and/or antioxidant enzyme levels increased in parallel with lipid peroxidation (e.g., Vider *et al.* 2001; Tauler *et al.* 2006). In regard to immune challenge, Torres & Velando (2007) also found that immunizing the blue-footed booby (*Sula nebouxii*) with lipopolysaccharide induced lipid peroxidation. To my knowledge, these two studies present the first direct evidence in wild animals

that immune system activation by a nonparasitic foreign antigen leads to lipid peroxidation.

Plasma carotenoids did not correlate with plasma antioxidativity, but carotenoid supplementation reduces lipid peroxidation (LPO) by 24%. Although this effect was not strong enough to compensate for the increased LPO due to immune challenge, it indicates that the antioxidant function of the carotenoids in avian models cannot be totally discounted, as suggested by some authors (Hartley & Kennedy 2004; Costantini & Moller 2008; Halliwell & Gutteridge 2007). This study also clearly showed that measuring antioxidant barrier alone is not sufficient to demonstrate the antioxidant function of carotenoids.

Although vitamin E is considered the main lipophilic antioxidant involved in membrane defence (Sies *et al.* 1992), vitamin E supplementation did not affect lipid peroxidation and any other parameters measured. It should be noted, however, that birds were fed sunflower seeds, which are known to be rich in vitamin E (Yoshida *et al.* 2002). It is known that higher doses of vitamin E produce relatively small increases in plasma tocopherol levels in humans (Burton *et al.* 1998) and barn swallows (de Ayala *et al.* 2006). Whether and how limiting nutrient vitamin E is in wild birds is largely unknown. On the other hand, it has been recently suggested (Halliwell and Gutteridge 2007) that vitamin E may have physiological effects other than antioxidant ones.

Notably, neither did vitamin E or carotenoid supplementation affect the strength of the swelling response to PHA. The association between carotenoids and immune function have been demonstrated in several avian studies (reviewed by McGraw *et al.* 2006), on the other hand, results demonstrating a lack of association have also started to accumulate (Navara & Hill 2003; McGraw & Ardia 2005; McGraw & Klasing 2006; McGraw *et al.* 2006). One reason for these inconsistencies may relate to the ways ecologist assess and interpret immunocompetence. For instance, PHA-induced skin swelling results from the activation of both adaptive and innate components of the immune system (Martin *et al.* 2006) that might be differentially affected by different antioxidants.

The result that levels of an endogenous antioxidant, uric acid, strongly contributed to plasma antioxidativity compares favourably with recent findings of Cohen *et al.* (2007), who demonstrated positive relationships between these variables in 92 wild bird species. Plasma uric acid has been shown to increase in parallel with lipid peroxidation in broiler chickens during chronic corticosterone exposure (Lin *et al.* 2004). It is known that urate levels go up in many human diseases because of alterations in purine metabolism and therefore could obscure depletions of other antioxidants (Halliwell and Gutteridge 2007). Additionally, evidence in humans suggests that proteins and micromolecular antioxidants such as ascorbate, polyphenols, and glutathione, can also importantly contribute to plasma antioxidativity (e.g., Erel 2004).

In conclusion, these results demonstrate the importance of antioxidant function of carotenoids in birds and show that simultaneous assessment of

oxidative stress-driven damage, antioxidant barriers, and individual antioxidants is critical for explaining the potential costs of immune system activation.

3.6. Does vitamin E supplementation affect plumage coloration? (VI)

As suggested previously, carotenoid-based signals have been an important model of honest signalling, as carotenoids are believed to play vital roles in several physiological functions including antioxidativity (**V**) and immunomodulation (**IV**), while they are also required for sexual displays. However, it has been recently suggested that carotenoids may be used mainly as signals, revealing the amount of non-pigmented antioxidants, which are more important biological protectants against free-radical-mediated oxidative stress. Thus, organisms may use carotenoids to advertise the amount of other, non-pigmented antioxidants rather than the antioxidative properties of carotenoids themselves (Hartley & Kennedy 2004). I tested this hypothesis by experimentally supplementing greenfinches with carotenoids and an uncoloured antioxidant, vitamin E, which is probably the most important agent in cell membrane defences as an inhibitor of the free radical chain reaction of lipid peroxidation (Fang *et al.* 2002; Sies *et al.* 1992). To estimate the effect of supplementation on plumage, the colour parameters of wild-grown and laboratory-grown tail feathers were measured.

Manipulation of dietary vitamin E availability did not affect the coloration of lab-grown feathers, although simultaneous carotenoid supplementation had significant positive effect on feather chroma. Lab-grown feathers of carotenoid supplemented birds had 30.6% higher chroma as compared to the feathers of unsupplemented individuals. These findings do not support the hypothesis that carotenoid-based ornaments serve mainly as indicators of the abundance of other, uncoloured antioxidants (Hartley & Kennedy 2004). In contrast, three other recent studies demonstrated that supplementary feeding with uncoloured antioxidants affected colouration. Melatonin supplementation resulted in enhancement of carotenoid-based bill coloration in captive male zebra finches (*Taeniopygia guttata*; Bertrand *et al.* 2006). Similarly, vitamin E and C supplementation resulted in enhancement of carotenoid-based integument coloration in sticklebacks (*Gasterosteus aculeatus*; Pike *et al.* 2007). Additionally, recent study by Perez *et al* (2008) demonstrated that in yellow-legged gull (*Larus michahellis*) vitamin E supplementation resulted in larger red spot on the lower mandible, but colour intensity was not affected by the supplements. These discrepancies can be probably explained either by the different antioxidants or by different ornaments and species studied. On the other hand, as discussed previously (**V**), sunflower seeds are rich in vitamin E (Yoshida *et al.* 2002). Thus, I cannot totally exclude the possibility that the effect of vitamin E supplementation in my study had no effect on feather coloration because all birds

received already the maximum absorbable concentration of tocopherols from their sunflower seed diet. However, at present it is largely unknown whether and how limiting nutrient vitamin E is in wild birds.

The finding that lab-grown feathers were all duller than wild-grown feathers, however, is not surprising and compares favourably with many previous studies (reviewed in Hill & McGraw 2006). Such a result can be most likely ascribed to insufficient carotenoids (or other micronutrients required for carotenoid biotransformations, transportations and deposition to integument) in the diet because sunflower seed diet is known to be a relatively poor source of carotenoids (McGraw *et al.* 2001). The result that increased carotenoid availability is mirrored in sexual ornaments is consistent with the current understanding of the mechanics of carotenoid-based signalling (e.g., Møller *et al.* 2000; Surai 2002). However, these results do not support the idea that carotenoids are mainly used as indicators of the abundance of other antioxidants.

SUMMARY

Estimation of individuals' parasite resistance and ability to alleviate the potential costs of immune activation is important for understanding the major forces shaping animals' life-histories and sexually selected traits. Yet the mechanisms by which individuals alleviate somatic costs associated with parasite infections and immunopathological damages are poorly known. It is widely believed that carotenoids play major roles in many important functions, including sexual signalling, immunomodulation and antioxidant protection. However, the exact physiological roles of carotenoids are not clear. The main aim of this thesis was to assess the relative importance of carotenoids and endogenous antioxidants in the context of expression of signal traits and immune function in greenfinch (*Carduelis chloris*) coccidiosis model.

Whether and by which mechanism could the coccidian infection affect carotenoid-based sexual ornamentation in greenfinches was investigated in paper **I**. Experimental infection with *Isospora lacazei* resulted in drastic effects upon the physiology and expression of carotenoid-based plumage colouration in greenfinches, probably due to the reduced absorption of nutrients (including carotenoids and vitamin E). This suggest that coccidian parasites provide an excellent mechanism for elucidating connection between the immune system and carotenoid-based ornament expression in greenfinches – infected birds with damaged digestive system are not able to deposit enough carotenoids into the feathers.

Variation in host resistance and parasite virulence within populations is an important assumption of models of parasite-mediated selection. This issue was tested in paper **II** by infecting greenfinches with Isosporan strains originating either from single or multiple hosts. The outcome of experimental infection with different Isosporan strains depended on concurrent variation in parasite virulence and host resistance. Infection with multiple strains resulted in greater virulence than single-strain infection, suggesting that parasites originating from different hosts are genetically diverse. It was also found that natural infection intensities confer information about the ability of individuals to resist novel strains. However, these findings suggest the great potential of avian coccidiosis as models for microevolutionary research.

In ecological studies, estimation of actual parasite resistance is often difficult, so researchers frequently rely on surrogate measures of immunocompetence. The question as to whether the standard immune tests reflect parasite resistance was tested in paper **III**. High humoral immune responsiveness indicated resistance to Isosporan parasites and better nutritional state but only among birds that were not vulnerable to novel infections. Strong cell-mediated immune responsiveness correlated positively with higher vulnerability to Isosporan infection – thus most "immunocompetent" individuals appeared least resistant to real infections. These examples call for caution when

interpreting the results of standard immune tests in the context of parasite resistance.

The question as to what exactly makes the activation of immune defences costly has remained poorly understood in the context of ecological research. The role of carotenoids in modulation the cost of immune activation and oxidative stress was investigated in paper **IV**. It was found that certain somatic costs associated with immune system activation can be alleviated by carotenoids, although measures of total antioxidativity did not correlate with plasma carotenoid levels. Humoral immune challenge suppressed the cell-mediated response, suggesting a trade-off between the uses of different arms of the immune system. However, I did not find that immune challenge had induced any damages that could be ascribed to oxidative stress because immune activation did not affect total antioxidant protection or carotenoid levels.

Whether activation of the immune system exacts oxidative costs and to what extent exogenous dietary antioxidants mitigate potential damage from free radicals was tested in paper **V**. Immune challenge caused lipid peroxidation but also elevated levels of total plasma antioxidativity. Carotenoid supplementation generally reduced lipid peroxidation but this did not compensate for the effects of immune activation. Vitamin E supplementation did not affect any of the studied parameters. I found no evidence that dietary antioxidants are immunostimulatory.

It has been recently suggested that carotenoid-based signals may be used to advertise the presence of other, uncoloured antioxidants rather than the antioxidative properties of carotenoids themselves. This hypothesis was tested in paper **VI** by supplementing carotenoids and vitamin E in greenfinches. Carotenoids – but not vitamin E – enhanced the coloration of the feathers. Thus, this result does not support the idea that carotenoids are mainly used as indicators of the abundance of other antioxidants.

In conclusion, the results of this thesis improve our understanding about the overwhelming complexity of the antioxidant protection and the role of carotenoids in immune function. They also call for cautious interpretation of potential costs of immune activation based on assessment of simple biochemical measurements. Identifying the exact mechanisms by which organisms cope with oxidative stress and alleviate the costs of immune activation remains a challenging task also for future studies of avian ecophysiology.

SUMMARY IN ESTONIAN

Antioksüdantne kaitse, karotenoidid ja koktsiidinakkus rohevintidel – immuunaktivatsiooni hinna ja parasiidiresistentsuse mehhanismide hindamine karotenoidsete ornamentidega värvulistel

Isendite parasiidiresistentsuse ja immuunaktivatsiooni kulukuse kompenseerimise mehhanismidest arusaamine on oluline mõistmaks loomade elukäiku ja sugulist valikut mõjutavaid evolutsioonilisi faktoreid. Küsimus sellest, kuidas kompenseeritakse parasiidinakkustest ja immunopatoloogilistest kahjustustest tulenevaid somaatilisi kulutusi, on paljuski segane. Eeldatavasti mängivad karotenoidid olulist rolli paljudes füsioloogilistes funktsioonides, kaasa arvatud signaaltunnuste ekspressioon, immunomodulatsioon ja antioksüdantne kaitse. Kuid milline on karotenoidide primaarne füsioloogiline roll, pole veel kaugeltki selge. Dissertatsiooni eesmärgiks oli hinnata karotenoidide ja endogeensete antioksüdantide suhtelist tähtsust signaaltunnuste väljaarendamise ja immuunfunktsiooni kontekstis rohevindi koktsidioosi mudelis.

Esimeses artiklis uuriti, kuidas ja mis mehhanismiga mõjutab koktsiidinakkus karotenoididel baseeruvate signaaltunnuste väljaarendamist rohevintidel. Eksperimentaalne nakatamine koktsiidiga *Isospora lacazei* mõjutas drastiliselt rohevintide füsioloogiat ja karotenoidset sulestiku värvust, tõenäoliselt toitainete (muu hulgas karotenoidid ja E-vitamiin) imendumise pärssimise tõttu. Saadud tulemus viitab sellele, et koktsiidinakkuse mudel võib pakkuda suurepärast mehhanismi isendi tervisliku seisundi ja karotenoidsete ornamentide seoste kirjeldamiseks – nakatunud ja sellest tulenevalt kahjustunud seedetraktiga linnud ei ole suutelised paigutama piisavalt palju karotenoide sulestikku. Kuna emased rohevindid eelistavad paarituda intensiivselt värvunud karotenoidsete ornamentidega isastega, siis võiks järeldada, et koktsidioosiresistentsus võiks olla üheks mehhanismiks, mis võimaldab isaste kvaliteedi ausat signaliseerimist sulestiku värvuse kaudu.

Parasiitide vahendatud sugulise valiku mudelid eeldavad populatsioonisisest varieeruvust nii peremehe resistentsuse kui ka parasiidi virulentsuse osas. Selle eelduse kehtivuse kontrollimiseks nakatati rohevinte koktsiidi *Isospora lacazei* erinevate tüvedega (**II**). Eksperimentaalse nakatamise tulemusena selgus, et mitme eri tüvega nakatamine põhjustas suurema virulentsuse kui üksiku tüvega nakatamine. Samuti leiti, et loomulik nakkusintensiivsus andis informatsiooni isendite vastupanuvõimest uute tüvede suhtes. Madala loodusliku nakkusfooniga isendid olid resistentsemad ka uuele segutüvedega nakatamisele. Saadud tulemused viitavad eksperimentaalse koktsiidinakkuse mudeli perspektiivikusele mikroevolutsioonilistes uuringutes.

Kuna tegeliku parasiidiresistentsuse hindamine on loomaökoloogilistes uuringutes keeruline, kasutatakse sellistes töödes enamasti kunstlikke immunokompetentsuse mõõtmise meetodeid. Kolmandas artiklis uuriti, kuidas stan-

dardsed immuuntestid peegeldavad parasiidiresistentsust. Kõrge humoraalne immunokompetentsus (antikehade produktsioon võõrerütrotsüütide vastu) peegeldas isendite koktsiidiresistentsust ja paremat toitumusseisundit, kuid ainult nendel lindudel, kes ei olnud vastuvõtlikud uuele nakkusele. Tugev rakuline immunokompetentsus (paistetusreaktsioon fütohemaglutiniini süstimisele) korreleerus aga positiivselt katseliselt indutseeritud koktsidioosi intensiivsusega – seega, fütohemaglutiniini testi järgi osutusid kõige immunokompetentsemad isendid reaalse patogeeni nakkuse suhtes kõige vähem resistentseks. Saadud tulemused kutsuvad üles standardsete immuunväljakutsete tulemuste kriitilisemale tõlgendamisele parasiidiresistentsuse kontekstis.

Millised mehhanismid konkreetselt tingivad immuunaktivatsiooni kulukuse, on jäänud ökoloogidele paljuski segaseks. Neljandas artiklis uuriti karotenoidide rolli immuunaktivatsiooni hinna ja oksüdatiivse stressi kompenseerimisel. Leiti, et karotenoidid võivad teatud ulatuses leevendada immuunaktivatsioonist tuleneva somaatiline hinna maksmist. Humoraalne immuunväljakutse pärssis rakulise immuunvastuse tugevust, viidates immuunsüsteemi eri komponentide vahel esinevale lõivsuhtele. Immuunväljakutse ei mõjutanud ei üldist antioksüdantset kaitset ega plasma karotenoidide kontsentratsiooni.

Millisel määral kutsub immuunaktivatsioon esile oksüdatiivse stressi ja mis ulatuses eksogeensed antioksüdandid pehmendavad vabade radikaalide tekitatud võimalike kahjustuste mõju, uuriti viiendas artiklis. Immuunväljakutse suurendas lipiidide peroksüdatsiooni, kuid samuti tõstis üldist plasma antioksüdantide taset. Lisatoitmine karotenoididega üldiselt vähendas lipiidide peroksüdatsiooni, kuid mitte sellisel määral, et kompenseerida immuunaktivatsiooni mõju. Eksogeensete antioksüdantide (karotenoidid ja E-vitamiin) immunostimuleeriv toime ei leidnud tõestamist.

Viimastel aastatel on levinud seisukoht, et karotenoidsed ornamendid signaliseerivad pigem teiste, mittepigmentsete antioksüdantide taset kui karotenoidide enda antioksüdatiivset potentsiaali. Kõnealust hüpoteesi testiti katses (vt kuues artikkel), milles rohevintidele manustati toidu kaudu karotenoide ja E-vitamiini. Lisatoitmine karotenoididega mõjutas sulestiku värviparameetreid positiivselt, ent E-vitamiin seda ei teinud. Seega ei toeta saadud tulemused hüpoteesi, mille järgi karotenoidid on peamiselt teiste antioksüdantide külluse indikaatorid.

Kokkuvõtteks: usun, et käesoleva dissertatsiooni tulemuste põhjal on võimalik paremini aru saada antioksüdatiivse kaitse ja karotenoidide olulisusest immuunfunktsioonis, samuti osutatakse siin biokeemiliste ja füsioloogiliste protsesside detailse mõistmise vajadusele evolutsioonilises ökoloogias. Siinsete tulemuste põhjal saab hinnata kriitilisemalt immuunaktivatsiooni võimalikku kulgu, tuginedes üksikute biokeemiliste parameetrite mõõtmise tulemustele. Küsimus sellest, mismoodi organismid rakendavad antioksüdatiivset kaitset oksüdatiivse stressi leevendamiseks, pakub jätkuvalt uurimisainet, milleks on vajalikud veel paljud ökofüsioloogiakatsed.

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I

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How coccidian parasites affect health and appearance of greenfinches

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Summary

1. The aim of this study was to examine the mechanisms by which parasites can affect the expression of ornamental traits.
2. Levels of an intestinal coccidian parasite, *Isospora lacazei*, were manipulated in captive male greenfinches (*Carduelis chloris*) by suppressing the natural infections with a coccidiostatic sulphonamide drug. Subsequently, half the birds were experimentally infected, while another half continued receiving medication.
3. Over the course of the experiment the effect of our treatments upon 14 mainly haemato-serological condition indices was recorded. Additionally, changes in colour and carotenoid content of yellow tail and breast feathers, which serve as sexually dimorphic ornamental traits, were measured.
4. Eighty-nine per cent of birds hosted chronic isosporan infection before the experiment, yet experimental inoculation with mixed parasite strains resulted in drastic but transient decreases in serum carotenoid, vitamin E, triglyceride and albumin concentrations, and reduced body mass, indicating serious pathology and probable nutrient malabsorption due to damaged intestinal epithelium.
5. Laboratory-grown tail feathers of infected birds contained 52% less carotenoids and also had smaller values of chroma and hue than those of medicated birds.
6. These results suggest that coccidian infection reduced the expression of plumage coloration by creating a deficiency of carotenoids available for deposition in ornamental feathers.

Key-words: carotenoids, coccidia, experimental infection, plumage colour, vitamin E.

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Introduction

Secondary sexual characters, such as the bright plumages of many bird species, are presumed to have evolved as signals of individual quality (Zahavi 1975). If the expression of such characters is sensitive to the health status of the bearer, individuals can signal their abilities to provide direct and/or genetic benefits to their prospective mates. The concept of parasite-mediated sexual selection proposes that only individuals most resistant to infections can express elaborate ornamentation because parasites withdraw resources that their hosts could otherwise invest into sexual display (Hamilton & Zuk 1982). Recently, it has been proposed that expression of different types of ornamental traits is suppressed by oxidative stress, generated in the process of extensive production of free radicals during immune responses (von Schantz *et al*. 1999). Among such traits, bright carotenoid-based integumental colours have been considered particularly sensitive to oxidative stress and host infection status (e.g. Lozano 1994).

Carotenoids participate in immuno-regulation and -stimulation, lymphocyte proliferation, free-radical scavenging and detoxification (review in Møller *et al*. 2000). Because animals cannot synthesize carotenoids (and hence have to acquire them from food), a trade-off

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between investment of carotenoids in maintenance and ornamentation can be predicted. Individuals that are forced to fight infections during the formation of carotenoid-based ornaments are expected to have less carotenoids available for developing colourful traits. (e.g. Lozano 1994; Olson & Owens 1998; Møller *et al*. 2000). However, despite the accumulating evidence on carotenoid-based sexual signalling, the exact mechanisms generating trade-offs between individual health status and signal expression are not completely understood (e.g. Olson & Owens 1998; Hill 1999). Therefore, to interpret the increasing number of correlative studies about the relationships between ornamental traits and parasite loads (reviewed in Møller *et al*. 2000), a detailed knowledge about the physiological processes underlying the effect of parasitism upon host health and appearance is needed. A valuable way to obtain such knowledge is by manipulating host infection status (through experimental infection and/or medication) and simultaneously monitoring the hosts' physiological condition and development of ornamental traits.

The aim of this study is to analyse the effect of coccidian infection upon health state and signal-trait expression in male greenfinches [*Carduelis chloris* (Linnaeus)]. Intestinal coccidians from the genus *Isospora* (Protozoa, Apicomplexa) infect a number of songbird species in the wild (reviewed by Giacomo *et al*. 1997; Duszynski, Couch & Upton 2000; McGraw & Hill 2000). Related species (coccidians from the genus *Eimeria*) are common parasites of poultry where they directly inhibit the uptake of essential dietary components, including carotenoids in the gastrointestinal tract of chickens (e.g. Allen 1987, 1997; Allen & Fetterer 2002a,b) and consequently depress carotenoid-based pigmentation of skin and legs ('pale bird syndrome'; Tyczkowski, Schaeffer & Hamilton 1991). Published evidence about the effects of coccidians upon carotenoid-based ornaments in wild species is more scarce since only two studies in carduelline finches have demonstrated that experimental infection with *Isospora* sp. causes birds to develop less saturated carotenoid-based plumage and bill coloration (Brawner, Hill & Sundermann 2000; McGraw & Hill 2000).

Greenfinches are medium-sized (*c*. 28 g) gregarious seed-eating passerines native to the western Palearctic region. Males are larger and more colourful than females, with old males developing olive-green plumage on the back, bright or greenish yellow colour in the breast, and striking bright yellow markings on the primaries, primary coverts and sides of the tail feathers. Females are more olive-brown and yellowish-buff, having faint brown streaks on back and lacking full yellow tints in their plumage (Cramp & Perrins 1994). The male plumage brightness (measured by visual scoring) has been shown to be a sexually selected trait, as more brightly coloured male greenfinches tend to be favoured by females as mates (Eley 1991). It has also been shown that males with more yellow ornamental feathers are less likely to be heavily infected with haemoparasites (Merilä, Sheldon & Lindström 1999) and have higher virus clearance rates (Lindström & Lundström 2000). The yellow pigments in the plumage of greenfinches consist of carotenoids, such as the canary xanthophylls (Stradi *et al*. 1995; Saks, McGraw & Hõrak 2003).

To describe the physiological processes accompanying coccidian infection in greenfinches, we applied various clinical screening tests including (i) estimates for total and differential leucocyte counts, (ii) serum protein concentrations and profiles, (iii) serum triglyceride concentration, (iv) serum concentrations of fat-soluble antioxidants such as carotenoids and vitamins E and A and (v) body mass. To estimate the effect of infections upon ornamental traits, we measured spectrophotometrically the colour of yellow tail and breast feathers and the carotenoid content of tail feathers. To our knowledge, this is the first time that such a diverse array of physiological and colourimetric measures have been used to estimate the impact of parasitism upon health and appearance in a wild animal species.

Materials and methods

A total of 28 male greenfinches were caught in mist-nets in the Vaibla bird station in Central Estonia (58°24′ N; 26°3′ E) between 2 and 20 October 2002. Birds were transported to Tartu and housed in individual indoor cages (27 × 51 × 55 cm). The study was carried out from 3 January (day 1) to 6 March (day 63). The birds were fed *ad libitum* with sunflower seeds and tap water. Sand and boiled chicken eggshell mixture was provided to the birds during a 1-week period (days 33–40 of experiment). During the study, birds were held on the natural day-length cycle. The study was conducted under the license from the Estonian Ministry of the Environment and the birds were released into their natural environment after the experiment.

RESEARCH PROTOCOL

On the morning (0700–0930 h) of day 1, all birds were blood-sampled to obtain individual, pre-experimental haemato-serological measurements (first time point in Figs 1 and 2). Two weeks after the first sampling, all the birds were treated against coccidia for 5 days (days 15–19) by adding Vetacox PLV (Sanofy-Synthelabo Inc., Paris, France) to the birds' drinking water (1 g of Vetacox dissolved in 2 L of water). Vetacox, like any other sulphonamide drug, is a broad-spectrum antimicrobial which is efficient against aerobic bacteria and protozoa. In microbial cells, sulphonamides interfere with the biosynthesis of folic acid (required for DNA synthesis) and thereby prevent multiplication of microbes (e.g. Appelgate 1983). Vetacox does not contain carotenoids or any other antioxidants, nor is there any reason to believe that it would interfere with carotenoid metabolism. We therefore assumed that all differences between medicated and infected birds arise entirely due to the antimicrobial action of the drug, rather than due

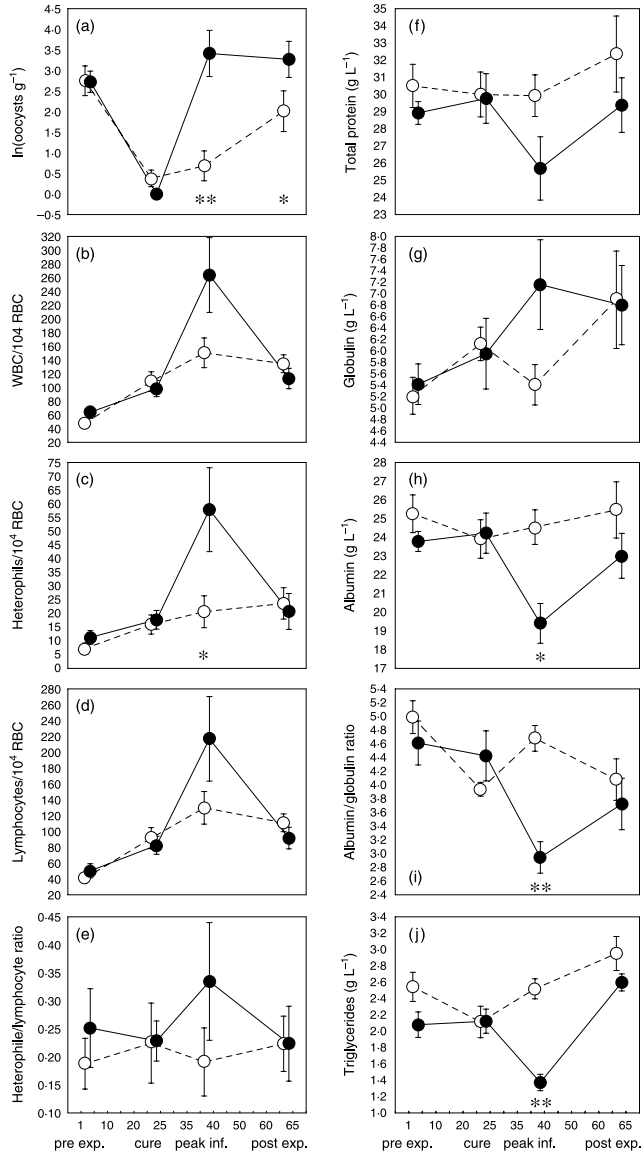

Fig. 1. Effect of manipulation of parasite load upon condition indices of greenfinches. Filled circles, infected group; empty circles, medicated group. Asterisks denote significance of pairwise contrasts between infected and medicated group (*$P < 0{\cdot}05$; **$P < 0{\cdot}001$). The course of experiment in days is noted in *x*-axes of the lower graphs. Sample sizes and statistics are given in Tables 1 and 2. Average individual changes in trait values (% of pre-experimental level) are shown in Appendix 3 (see Supplementary material).

to boosting of any other physiological aspect of drug-treated birds.

On the second day of anticoccidial medication (day 16), wild-grown feathers from the breast and tail of the birds were collected for colour measurements. On the morning of day 21 (when the parasite loads of all birds reached zero), the second set of blood samples was taken (time point 'cure' in Figs 1 and 2). A week later

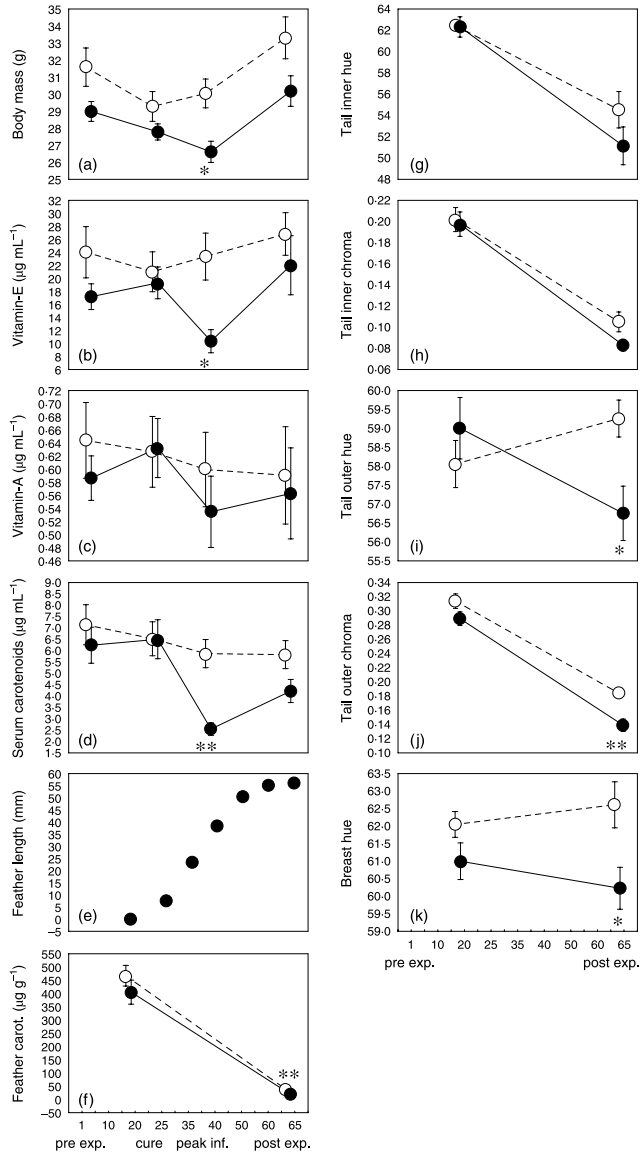

Fig. 2. Effect of manipulation of parasite load upon condition indices and feather coloration of greenfinches. Filled circles, infected group; empty circles, medicated group. Asterisks denote significance of pairwise contrasts between infected and medicated group (*$P < 0{\cdot}05$; **$P < 0{\cdot}001$). The course of experiment in days is noted in *x*-axes of the lower graphs. Sample sizes and statistics are given in Tables 1 and 2. Average individual changes in trait values (% of pre-experimental level) are shown in Appendix 3 (see Supplementary material).

(on day 28), 14 randomly selected birds comprising the infected group were inoculated orally with 2000 sporulated oocysts of *Isospora lacazei* (Labbe). Concurrently, the 14 individuals from the medicated group were subjected to a 5-day medication period (day 28–32) with Vetacox. Hereafter, the birds in the medicated group received further medication with Vetacox in 5-day periods with 2-day intervals between the periods,

until day 52 (medication lasted from days 35–39, 42–46 and 49–53). Eight days after the experimental treatment (drug treatment vs. infection; day 36) the third set ('infection/cure') of blood samples was taken. On day 63 (last time point in Figs 1 and 2), the laboratory-grown feathers had reached their full length and were collected. On the morning of the same day, the last set of blood samples (post-experiment) was also collected.

To assess endoparasite loads, we collected faecal samples during 3 days around the blood samplings (days 1–3, 19–21, 34–36 and 61–63). The individual growth rate of laboratory-grown tail feathers was recorded with the weekly interval, starting from 11 days (day 27) after the removal of the wild-grown tail feathers (Fig. 2e).

We did not use a third control group of totally untreated birds because it appeared impossible to maintain greenfinches in captivity as parasite-free without anticoccidial treatment. A pilot study showed that greenfinches kept in captivity without treatment become as intensively infected (due most probably to relapse of chronic latent infections) as the birds which were experimentally infected with 2000 oocysts in the current experiment. Reference values of 15 mainly haemato-serological condition indices of greenfinches not treated against coccidia have been reported by Hõrak *et al*. (2002 and 2003).

PARASITES

Coccidia of the genus *Isospora* are obligate intracellular parasitic protozoa, following the typical apicomplexan life cycle. A host becomes infected when it ingests oocysts that have been passed in the faeces of another host. The oocyst excysts in the epithelial cells of intestinal mucosa and liberates sporozoites from its contents. The sporozoites penetrate the cells of the host's small intestine and reproduce asexually. In case of passerine birds, the first-generation sporozoites may also invade liver, spleen and lungs (atoxoplasmosis, e.g. Giacomo *et al*. 1997). In the epithelial cells of intestine, each generation of asexual reproduction produces multiple merozoites that infect new cells. This stage of the infection can result in destruction of massive numbers of cells in the host's small intestine and, ultimately, lead to the host's death (e.g. Box 1977; Sironi 1994). Some of the merozoites that enter the host's cells transform into gametocytes. The gametocytes transform into gametes, the gametes fuse, and the resulting zygote begins to develop into an oocyst. The developing oocyst escapes from the host's cell, and it is passed in the host's faeces.

To identify the coccidia infecting greenfinches, 60 oocysts, taken from a mix of oocysts collected from the eight individuals, were examined with phase contrast microscope (magnifications 40×15 and 90×10 with oil immersion). Species was determined as *Isospora lacazei* according to Scholtyseck (1979) and Levine (1982). Isosporan oocysts to be used for oral inoculation were collected from the faecal samples of eight birds that had heavy natural infections during the 5-day period before the experiment. Following collection, faeces were mixed with tap water in a ratio of 2 mL of water per 1 g of faeces. The mixture was held at room temperature for 1 h. Consecutively, the mixture was drained through gauze into separate 10 mL polypropylene tubes. Oocysts were separated from faecal particles by three successive washing procedures with 10 mL of water by centrifugation at 2500 r.p.m. for 7 min. During each washing procedure, the supernatant was removed after centrifugation, leaving 1 mL residue with oocysts. After the third washing the oocysts were floated by dissolving the 1 mL residue into 10 mL of a 45% table sugar (saccharose) solution. This solution was centrifuged at 3000 r.p.m. for 15 min. Then, a 0·5-mL aliquot was collected from the surface of the solution, where the oocysts were floating, and diluted in 10 mL of water. The sugar was removed from this solution by washing the oocysts with water. This procedure gave us clean oocysts, suspended in 1 mL of water. Oocysts were preserved in 20 mL of 2% potassium dichromate ($K_2Cr_2O_7$) solution at room temperature and aerated daily. Sporulation of the oocysts was registered 5 days after collecting by microscopic observation. For the experimental infestation, the oocysts were washed with water to remove potassium dichromate from the solution. The birds of the infected group were inoculated orally with 2000 oocysts diluted in 120 µL of water.

To determine individual parasite loads, individual faecal samples were collected around 1800 h (due to diel periodicity in oocyst shedding; see, e.g. Brown, Ball & Holman 2001). The collected faecal samples were weighed to the nearest 0·01 g with an electronic balance (Mettler Toledo AB-S, Greifensee, Switzerland), suspended in 1 mL of water and held at room temperature for 30 min. Then, the solution was drained through gauze into individual tubes and centrifuged at 1500 r.p.m. for 7 min. The supernatant was removed and 0·2 mL of saturated NaCl water solution was added to the 0·5 mL residue. The number of oocysts was counted using the Goryayev haemocytometer (volume = 0·0018 mL) and their concentration was expressed as number of oocysts per gram of faecal sample. The average of two counts from individual faecal samples was used as the estimate of daily oocyst production rate. The averages of parasite counts of 3 days, preceding the blood-sampling days (days 1, 21, 36 and 63), were used in statistical analyses. This was carried out to account for possible daily variation in individual oocyst counts and also because on day 21 all individual samples appeared parasite-free, which would have not permitted the use of repeated-measures ANOVA for analysis of variation in individual parasite load.

LEUCOCYTE COUNTS

For counting leucocytes, a drop of blood was smeared onto two individually marked microscope slides,

air-dried, fixed in absolute methanol and stained with azure-eosin (Reachim, Chostkinsk, Russian Federation). The proportion of different types of leucocytes was assessed on the basis of examination of 100 leucocytes under 1000 × magnification under oil immersion. Estimates of the total white blood cell count (WBC) were obtained by counting the number of leucocytes per approximately 10 000 erythrocytes. Differential leucocyte counts were obtained by multiplying their proportions with WBC. The repeatabilities of leucocyte counts obtained in this method were found to be reasonably high and significant (Ots, Murumägi & Hõrak 1998).

SEROLOGICAL ANALYSES

Total plasma protein concentration was determined in a photometric colourimetric test using the Biuret method. Standard agarose gel electrophoresis with the REP System (Helena Laboratories, Beaumont, TX, USA) was used for detection of major protein groups (see Ots *et al*. 1998 for details). Due to difficulties in separating the pre-albumin fraction from albumin, summed concentrations of both are reported and termed albumin concentration. Serum triglyceride concentrations were determined by enzymatic colourimetric test (GPO-PAP) using Biosub ® TG kit (Biocon ®, Germany).

Vitamins A, E and carotenoids were extracted from plasma with hexane after protein precipitation with ethanol as described previously by Surai *et al.* (2001). Vitamins E (α-tocopherol) and A (retinol) were determined as described by Surai, Noble & Speake (1996) using a high performnce liquid chromatography (HPLC) system (Shimadzu Liquid Chromatograph, LC-10AD, Japan Spectroscopic Co., Ltd, with JASCO Intelligent Spectrofluorometer 821-FP) fitted with a Spherisorb, type S30DS2, 3μ C18 reverse phase HPLC column, 15 cm × 4·6 mm (Phase Separations Ltd, UK). Chromatography was performed using a mobile phase of methanol/water (97 : 3, v/v) at a flow rate of 1·05 mL min^{-1} with a fluorescence detection of α-tocopherol and retinol. Calibration was performed using α-tocopherol and retinol solutions in methanol. Tocol was used as an internal standard. Total carotenoids were determined from the same extract using the same HPLC system, but fitted with a Spherisorb, type S5NH2 5μ C18 reverse phase HPLC column, 25 cm × 4·6 mm (Phase Separations Ltd, UK). Chromatography was performed using a mobile phase of methanol/water (97 : 3, v/v) at a flow rate of 1·5 mL min^{-1}. Total carotenoids were detected at 445 nm as a single peak using lutein as a standard.

FEATHER COLORATION

For the analysis of plumage colour, two to four breast feathers and one, right outermost (6th) tail feather was collected from each individual. In the absence of a feather on the right side of the tail (in three cases) the feather sample was taken from the left side of the tail. The wild-grown breast feathers were collected from the standard position: the midpoint between the middle-part of the sternum and the edge of the wing. Laboratory-grown feathers were collected from the same positions. Collected feathers were placed into a plastic bag and stored in the dark until measurements were carried out. Locations of colour measurements are indicated in Appendix 1 (see Supplementary material). Colour was measured from the feathers placed on a black background, in an area of approximately 1 mm^2, of the visible surface of the feather, using a spectrophotometer (Ocean Optics S2000) as described by Saks *et al*. (2003). To estimate colour, we calculated values of hue and chroma (see Endler 1990 for details). Hue can be understood as the everyday meaning for colour (yellow, green, red, etc.). Hue is a correlate of the shape of the reflectance spectrum, measured in degrees, around a circular spectrum (colour wheel) whereas chroma is a measure of the 'purity' or 'saturation' of a colour. Details for calculations of colour parameters are given in Saks *et al*. (2003). The repeatabilities (Lessells & Boag 1987) of different colour measurements averaged at 0·74 ± 0·12 (SD) and ranged from 0·50 to 0·88 (Appendix 2; see Supplementary material). In analyses, we use values of hue and chroma for tail feathers and only hue for breast feathers because the chroma of the one set of breast feathers (wild-grown, r between two different pairs of feathers) was too poorly repeatable (r = 0·35). As shown previously in the same set of feather samples, hue and chroma appeared good indicators of feather carotenoid content. Variation in the average carotenoid concentration of the yellow area of tail feathers explained 32–51% of the variation of in these colour parameters (Saks *et al.* 2003).

FEATHER CAROTENOID CONTENT

The yellow pigmented portions of the sampled feathers were trimmed off and weighed to the nearest 0·00001 g with an electronic balance (Mettler Toledo AG245, Greifensee, Switzerland). The carotenoids were extracted from the feather samples using acidified pyridine (Hudon & Brush 1992), purified, and identified with HPLC as described in Saks *et al*. (2003). The total carotenoid concentration (μg g^{-1}) in each sample was determined by spectrophotometry (Bausch and Lomb Spectronic 1001).

STATISTICS

The effect of experimental manipulation of coccidian infection in greenfinches was examined with repeated-measures ANOVA, assuming that the effect of treatment will be revealed by a significant 'time × treatment' interaction term. Additionally, we examined the differences between infected and medicated birds by calculation of pairwise contrasts between group averages during peak infection phase and post-experimental phase. Significant differences are indicated by asterisks in Figs 1 and 2; detailed statistics are presented in Table 2. In the case

of three variables (lymphocyte count, body mass, vitamin A concentration) the assumption of sphericity of variance–covariance matrix was violated. In these cases, the significance values were based on Geisser–Greenhouse correction. To obtain normal distribution of variables or homogeneity of variances (according to Levene's test), values for parasite count, WBC, heterophile count and serum globulin concentration were log-transformed. However, data for all variables (except for parasite counts) are presented as untransformed in Figs 1 and 2 in order to provide reference data. Data are presented as mean ± SD; all *P*-values are for two-tailed tests. Sample sizes vary between analyses due to our inability to obtain all required parameters from all birds throughout the study. This holds especially for fat-soluble antioxidants where the sample size (eight medicated and eight infected birds) is considerably smaller than that for the rest of variables.

Results

In the pre-experimental period, 89% (25/28) of all individuals were infected with coccidia. The average intensity of infection was 3595 ± 8231 oocysts g^{-1}. After 5 days' treatment with a coccidiostat, the average parasite loads in both experimental and control groups dropped to pre-experimental levels (Fig. 1a), with all faecal samples appearing parasite-free on day 6 after the start of medication. Eight days after infecting the experimental group (and continued drug treatment in the control group), the average difference in oocyst output between two bird categories was 263 times (infected 87812 ± 123347; medicated 334 ± 674). Oocyst output of infected group in the peak infection phase rose 16 times ($P = 0{\cdot}010$; $Z = 2{\cdot}55$; Wilcoxon's matched pairs test) above the pre-experimental level (of 1691 ± 1920 oocysts g^{-1}). Parasite load in the infected group remained high for 35 days following experimental infection (fourth time-point in Fig. 1a), while in the control group the parasite load started to increase slowly after withdrawal of medication. On the 10th day after the last medication, 50% of the medicated birds were already shedding oocysts. Group average oocyst output by that time was 4129 ± 9108, which approached pre-experimental levels (5363 ± 11185). Hence, our experiment resulted in successful manipulation of coccidian parasitism in greenfinches, the differences in infection intensity between experimental categories being highest in the third time point of sampling (Table 1, Fig. 1a). In that period (termed hereafter as peak-infection phase), the experimental group had been infected for 8 days while the control group was continuously receiving medication.

Based on significant 'time × treatment' interaction terms in Table 1, the following physiological parameters were affected by the experimental manipulation of coccidian parasite load. The peak-infection phase was followed by a transient increase in total leucocyte count (Fig. 1b), which was due mainly to heterophils (infected birds had a 36% higher concentration of circulating heterophils than medicated birds; Fig. 1c). The increase in lymphocyte count in our experimental group (Fig. 1d) was not significant (Table 1), nor did the treatment significantly affect heterophile/lymphocyte ratio (Fig. 1e). Total serum protein (Fig. 1f) and globulin concentration were not significantly affected by the treatment (although a tendency for increased globulin levels in the infected group is suggested by inspection of Fig. 1g). Serum albumin content was 24% lower among infected birds than controls in the peak infection phase (Fig. 1h). This drop in serum albumin evidently led to a significantly lower albumin/globulin ratio in the infected group in the peak infection phase (Fig. 1i). Similarly to that of albumin, serum triglyceride concentration in the infected group also decreased during peak infection, being 34% lower than pre-infection level and 46% lower than in the control group (Fig. 1j).

Average body mass in infected group was consistently lower than in the medicated group (Fig. 2a); however, pairwise contrasts indicated a significant difference only in the peak infection phase ($F_{1,25} = 10{\cdot}4$, $P = 0{\cdot}004$). Thus, by that time infected birds had lost on average of 2·4 ± 3·0 g (i.e. 8%) of their pre-experimental mass, while the medicated birds had lost on average 1·5 ± 1·8 g (i.e. 5%), suggesting that experimental infection had also an effect on body mass in greenfinches. During the peak infection phase, infected birds had 40% lower serum vitamin E concentrations than in the pre-experimental period and 56% lower vitamin E levels than in the medicated group (Fig. 2b). Vitamin A concentrations were not obviously affected by the experimental procedure (Fig. 2c). Serum carotenoid concentration (Fig. 2d) in the infected group dropped abruptly during the peak infection (59% compared to initial levels and 57% compared to medicated group).

Feather moult (Fig. 2e) occurred at a time when the medicated group received continuous medication and the infected group remained heavily infected (Fig. 1a). Compared to wild-grown feathers, the average carotenoid content of laboratory-grown feathers was 93% lower than initial in both groups of birds. Nevertheless, the total carotenoid content by the end of experiment was significantly (52%) lower in the infected compared to the medicated group (19·1 ± 10·9 μg g^{-1} vs. 39·8 ± 15·6 μg g^{-1}; Fig. 2f).

We found no differences between the treatment groups with respect to the colour of the inner vane of tail feathers (Fig. 2g,h). The coloration of the outer vane was obviously more affected by the treatment because its hue decreased by 4% (as compared to wild-grown feathers) in the infected group, while in the medicated group the value of hue increased by 2% compared to the initial (Fig. 2i). As a result, laboratory-grown feathers of infected birds had 4% lower values of hue than those of medicated birds. A significant effect of manipulation was also manifested by a significant 'time × treatment' interaction term in Table 1. The chroma of the outer vane was consistently higher (i.e. among both wild- and laboratory-grown feathers) in the group of medicated

Table 1. Effect of manipulation of parasite load upon condition indices and plumage coloration of greenfinches. Direction of the effects is presented in Figs 1 and 2. Med. and Inf. stand for sample sizes of medicated and infected group, respectively

Trait	Type of response	*F*	*P*	*n*
Parasite load	treatment	11·95	0·002	Med. 13
	time	25·38	< 0·00001	Inf. 14
	time × treatment	10·03	0·00001	
WBC	treatment	0·52	0·482	Med. 8
	time	38·66	< 0·00001	Inf. 8
	time × treatment	4·30	0·0099	
Heterophil count	treatment	1·78	0·203	Med. 8
	time	14·37	< 0·00001	Inf. 8
	time × treatment	4·02	0·013	
Lymphocyte count	treatment	0·70	0·418	Med. 8
	time	14·38	0·0009	Inf. 8
	time × treatment	2·61	0·121	
Heterophil/lymphocyte ratio	treatment	0·78	0·393	Med. 8
	time	0·23	0·873	Inf. 8
	time × treatment	0·66	0·582	
Total protein concentration	treatment	2·12	0·158	Med. 14
	time	2·26	0·089	Inf. 13
	time × treatment	1·03	0·383	
Albumin concentration	treatment	3·60	0·070	Med. 14
	time	3·92	0·012	Inf. 12
	time × treatment	3·75	0·015	
Globulin concentration	treatment	0·42	0·523	Med. 14
	time	2·91	0·040	Inf. 12
	time × treatment	1·69	0·178	
Albumin/globulin ratio	treatment	3·55	0·072	Med. 14
	time	7·55	0·0002	Inf. 12
	time × treatment	7·997	0·0001	
Triglyceride concentration	treatment	6·16	0·020	Med. 13
	time	38·61	< 0·00001	Inf. 13
	time × treatment	17·65	< 0·00001	
Body mass	treatment	5·73	0·025	Med. 14
	time	24·18	< 0·00001	Inf. 13
	time × treatment	1·69	0·195	
Vitamin E concentration	treatment	3·18	0·096	Med. 8
	time	4·18	0·011	Inf. 8
	time × treatment	2·48	0·074	
Vitamin A concentration	treatment	0·26	0·615	Med. 8
	time	1·69	0·203	Inf. 8
	time × treatment	0·49	0·622	
Plasma carotenoid concentration	treatment	2·84	0·114	Med. 8
	time	21·73	< 0·00001	Inf. 8
	time × treatment	7·44	0·0004	
Feather carotenoid concentration	treatment	1·88	0·183	Med. 13
	time	183·84	< 0·00001	Inf. 12
	time × treatment	0·48	0·497	
Tail inner hue	treatment	2·03	0·168	Med. 13
	time	44·56	< 0·00001	Inf. 11
	time × treatment	1·32	0·263	
Tail inner chroma	treatment	1·87	0·186	Med. 13
	time	107·22	< 0·00001	Inf. 11
	time × treatment	0·75	0·396	
Tail outer hue	treatment	1·45	0·241	Med. 13
	time	0·60	0·448	Inf. 12
	time × treatment	6·49	0·018	
Tail outer chroma	treatment	23·25	0·00007	Med. 13
	time	194·61	< 0·00001	Inf. 12
	time × treatment	1·03	0·320	
Hue of breast feathers	treatment	6·63	0·017	Med. 14
	time	0·07	0·792	Inf. 12
	time × treatment	2·77	0·109	

Table 2. Pairwise contrasts (from ANOVA) of condition indices between infected and medicated birds during peak infection phase and of colour measurements after the experiment. Significant differences between averages of drug-treated and infected birds are also indicated by asterisks in Figs 1 and 2

Trait	d.f.	*F*	*P*
Parasite load	1; 25	21·92	0·00009
WBC	1; 14	4·29	0·057
Heterophil count	1; 14	7·10	0·019
Lymphocyte count	1; 14	2·16	0·164
Heterophil/lymphocyte ratio	1; 14	1·41	0·256
Total protein concentration	1; 25	3·78	0·063
Albumin concentration	1; 24	13·48	0·001
Globulin concentration	1; 24	3·62	0·069
Albumin/globulin ratio	1; 24	34·98	< 0·00001
Triglyceride concentration	1; 24	52·23	< 0·00001
Body mass	1; 25	10·42	0·004
Vitamin-E concentration	1; 14	10·48	0·006
Vitamin-A concentration	1; 14	0·67	0·425
Plasma carotenoid conc.	1; 14	23·99	0·0002
Feather carotenoid conc.	1; 23	14·44	0·0009
Tail inner hue	1; 22	1·88	0·184
Tail inner chroma	1; 22	3·73	0·067
Tail outer hue	1; 23	8·59	0·008
Tail outer chroma	1; 23	19·48	0·0002
Hue of breast feathers	1; 24	6·95	0·014

birds (Fig. 2j). However, inspection of pairwise contrasts revealed that wild-grown feathers did not differ significantly between the groups ($F_{1;23} = 3·0$; $P = 0·095$), while among laboratory-grown feathers, infected birds had significantly lower values of chroma than medicated birds ($F_{1;23} = 19·5$; $P = 0·0002$; 22% difference). Similar to the results for outer-vane chroma, the hue of the breast feathers was, in both cases, higher in the group of medicated birds (Fig. 2k). Again, pairwise contrasts indicated that wild-grown feathers did not differ significantly between the groups ($F_{1;24} = 2·8$; $P = 0·105$), while among laboratory-grown feathers, infected birds had significantly lower values of hue than medicated birds ($F_{1;24} = 7·0$; $P = 0·014$; 4% difference).

Discussion

EFFECT OF INFECTION ON HOST PHYSIOLOGY

Our experimental medication and infection protocol with coccidian parasites resulted in successful manipulation of parasite oocyst output in captive greenfinches. Prevalence of isosporan oocysts dropped from 89% of pre-experimental level to 0% 6 days after the start of medication. It should be noted, however, that this medication did not result in complete clearance of parasites, as by the 10th day after withdrawal of medication, 50% of the medicated birds had again started to shed parasitic oocysts. This is not surprising, because the action of sulphonamide drugs is coccidiostatic, i.e. based upon the prevention of the multiplication of microbes. However, the effects of medication were detectable for at least 10 days after withdrawal because in the final sampling point of the experiment (35 days after infection of the experimental group and 10 days after withdrawal of medication in the medicated group), significant differences in average oocyst loads of two bird categories were still present (Fig. 1a). Most importantly, the treatment with Vetacox was sufficient to cause more than a 200-fold difference in oocyst output between medicated and infected groups during the peak infection phase. Thus we believe that the differences in physiology and appearance between our experimental groups emerged due to suppression of coccidian multiplication in drug-treated birds. This is also indicated by the clinical profile of infected birds, which is typical of intestinal infection.

Among the most conspicuous symptoms accompanying the peak phase of infection were the sudden and transient decreases of serum albumin, triglyceride, vitamin E and carotenoid concentrations (Figs 1h,j and 2b,d). The most probable explanation for these parallel patterns is the reduction of digestive and absorptive capacity of mucosa, which follows the destruction of epithelial cells during the reproduction of the parasite (e.g. Ruff & Fuller 1975; Hoste 2001). Isosporan parasites of cardueline finches have been shown to damage most extensively the duodenal and jejunal part of the intestine (Giacomo *et al.* 1997). In domestic chicken (Turk 1974), these intestinal compartments have been shown to be responsible for absorption of proteins (jejunum) and fats (duodenum), as well as vitamin E and carotenoids (Surai 2002), which would explain the drop in serum albumin, triglycerides and fat-soluble antioxidants (carotenoids and vitamin E). In humans and domestic chickens, lipid malabsorption causes decreases in plasma carotenoids and is a common cause of decreased plasma vitamin E, leading to deficiency syndromes (reviewed in Allen & Fetterer 2002a). A recent study on chickens infected with *Eimeria maxima* showed that the reductions in plasma vitamin E (and carotenoid) levels during acute phase of infection depended on the dose of inoculation and that providing high dietary levels of vitamin E did not afford any antioxidant protection to hosts (Allen & Fetterer 2002a). With regard to serum albumin, the reduction of its concentration during the peak infection phase could be caused additionally by leakage of this protein into the intestinal lumen because of the damaged mucosa (Allen & Fetterer 2002a). Altogether, these infection-induced effects upon host nutritional status were evidently severe enough to lead to significantly lower body mass in the infected compared to the medicated group during the peak infection phase (Fig. 2a). Furthermore, an additional reason for the drop of serum carotenoids and vitamin E during the peak infection phase could be due to their usage in antioxidant defence against free radicals produced during the immune response (e.g. Allen 1997). For instance, coccidians may stimulate phagocytic cells of the host's immune system to produce superoxide anion that by itself, or as an initiator of free radical cascades, causes pathology and oxidizes carotenoids

(Allen, Danforth & Levander 1997) and/or depletes vitamin E storages (Allen & Fetterer 2002a). Yet another reason for the reduction in blood carotenoid levels due to infection may relate to depressed synthesis of high density lipoproteins (the main carriers of carotenoids and vitamin E) in the intestinal mucosa, as has been shown during *E. acervulina* infections in chickens (Allen 1987). These pathways, however, would not explain the reductions in body mass or serum albumin and triglyceride levels during the peak infection phase. Notably, with respect to the effect of coccidian infection upon the body mass of birds, our results resemble two other studies of cardueline finches (greenfinches, Cooper *et al*. 1989 and American goldfinches, McGraw & Hill 2000). In the latter study, infected birds also reduced food consumption, suggesting that infection-induced anorexia may play an additional role in the loss of body mass due to coccidiosis (see also Allen 1987).

With regard to the effect of infection upon leucocyte counts, our results differ from those of Rose, Hesketh & Ogilvie (1979) where primary eimerian infections in chickens and rats resulted in a biphasic increase in leucocyte (both heterophile and lymphocyte) count with intermittent lymphopenia during the peak infection phase. In greenfinches, however, only a marked heterophilia could be detected (Fig. 1c). A possible reason for this difference is that animals in the study of Rose and colleagues were immunologically naive to eimerian parasites, while 89% of greenfinches naturally hosted chronic isosporan infections. Our birds were reinfected later with a mixture of parasitic strains from different hosts, which is expected to induce different immune responses than occurred during primary infections. The increase of peripheral heterophil count in greenfinches may be associated with increased traffic of these phagocytozing cells to the intestinal mucosa, where they participate in the removal of debris (e.g. destroyed host tissues, dead parasites; e.g. Rose *et al*. 1979). Such phagocytotic processes are often linked to remarkable tissue damage caused by the production of proteolytic enzymes and free radicals (e.g. Klasing & Leshchinsky 1999). Notably, the pathogenicity of isosporan parasites in black siskins (*Carduelis atrata*) has been associated with abnormal and inadequate cell-mediated responses to parasites (Giacomo *et al*. 1997).

To sum up, the changes in host physiology, similar to those observed in this study, have been recorded previously in domestic chicken infected with various species of *Eimeria* (e.g. Ruff & Fuller 1975; Conway *et al*. 1993; Allen & Fetterer 2002a). Our study provides the first evidence that isosporans of wild birds (whose biology differs from that of *Eimeria* species) can cause similar physiological effects as eimerian parasites in poultry. Furthermore, this study outlines the specific diagnostic values of serum albumin, triglycerides, carotenoids and vitamin E as a markers of the severity of isosporan infection. These markers would be useful in future studies examining the pathogenicity of isosporan parasites in experimental infection protocols.

EFFECT OF INFECTION ON FEATHER COLOUR AND CAROTENOID CONTENT

The laboratory-grown tail feathers of infected birds contained 52% less carotenoids than those of medicated birds, and their outer vanes had 22% smaller values of chroma and 4% smaller values of hue than medicated birds (Fig. 2f,i,j). Similarly, the laboratory-grown breast feathers of infected birds had 4% smaller values of hue than medicated birds (Fig. 2k). Therefore, we can conclude that experimental coccidian infection indeed affected feather carotenoid content and colour in captive greenfinches. Hence, our results present direct experimental support for one of the basic premises of parasite-mediated sexual selection, namely that parasites affect the expression of secondary sexual traits. Along with the studies of two other cardueline finches (Brawner *et al*. 2000; McGraw & Hill 2000), this is another piece of evidence illustrating the modification of carotenoid-based plumage colour by coccidian parasites. More generally, our results are in line with those of experimental infections of other taxa. For instance, experimentally parasitized male sticklebacks (*Gasterosteus aculeatus*; Milinski & Bakker 1990) and guppies (*Poecilia reticulata*; Houde & Torio 1992) developed reduced carotenoid-based breeding coloration. Similarly, male laboratory mice (*Mus musculus*) infected with coccidian parasite *E. vermiformis* lost the attractiveness of the odour of their urine for the females (Kavaliers & Colwell 1995).

DECLINE IN COLOUR AND CAROTENOIDS INDEPENDENTLY OF INFECTION

Besides the effect of experimental infection it should be noted, however, that experimentally induced differences in both feather carotenoid content and colour were relatively small compared to differences between wild-grown and laboratory-grown feathers. On average, laboratory-grown tail feathers of both infected and medicated birds contained 93% less carotenoids than wild-grown feathers (Fig. 2f), while the chroma of outer and inner vanes of tail feathers decreased, respectively, 47% and 50% (Fig. 2j,h) and hue of the inner vane decreased by 15% (Fig. 2g) in captivity. Hence, some factor related to captivity (or season) *per se* affected feather carotenoid content and some components of colour much more strongly than coccidian infection. Which factors could account for this effect? We can see two most likely (not mutually exclusive) explanations here.

First, the pure sunflower seed diet used in this study might have been an insufficient source of dietary carotenoids required for the development of bright yellow plumage. The total concentration of carotenoids in sunflower seeds is about 1 $\mu g\, g^{-1}$; the main carotenoid is lutein (65% of total), followed by 34% of zeaxanthin and 2% of β-carotene (McGraw *et al*. 2001). This is generally thought to be low for most edible plant parts

(Goodwin 1980); in fact, other common bird seeds have 4–5 times more carotenoids than this (e.g. white millet; McGraw *et al*. 2001). Fed a low concentration of dietary carotenoids, captive greenfinches in this study accumulated low levels of serum carotenoids (6–7 μg mL^{-1}; Fig. 2d), which probably contributed to their faded plumage colour. A study of a closely related species, the American goldfinch (*C. tristis*), showed that wild-caught goldfinches circulate nearly two to three times the concentration of carotenoids in blood (15–30 μg mL^{-1}) than do captive birds fed a sunflower seed diet during moult and protected from coccidian infections (6–9 μg mL^{-1}; Gregory 2002). We cannot test this idea adequately in greenfinches, however, because we are currently not aware of the natural carotenoid concentration in the blood of greenfinches during moult.

Secondly, even at the level of sufficient carotenoid content, the diet of sunflower seeds might have been poor in some other essential micronutrients required for biotransformation, transportation and deposition of carotenoids in the feathers. For instance, the levels of β-carotene and vitamin A are very low in sunflower seeds (Harper & Skinner 1998; McGraw *et al*. 2001), while the levels of riboflavin (vitamin B_2) are considered to be relatively low in seeds (Harper & Skinner 1998). The passage of carotenoids from food to feathers involves multiple steps and mediators, such as substrate-specific membrane proteins in the gut, carrier proteins for transportation and enzymes for bioconversion. As pointed out by Hill (2000), the details of all these processes remain virtually unstudied but clearly there exists the potential for nutritional stress, independent of carotenoid availability to affect plumage coloration of birds. The best example for this is the study of Hill (2000) where food-restricted male house finches (*Carpodacus mexicanus*) grew less red plumage than *ad libitum*-fed controls, despite receiving equal carotenoid doses in their diet.

Ultimately, it is important to recognize that our study reflects the relationship between health and appearance of greenfinches under captive conditions, where carotenoid access and status was much lower than in the wild. There is evidence in a congeneric finch, the American goldfinch, that carotenoid-based coloration in the wild is linked correlationally to coccidial burdens as it was in our study (Olson 1996), but whether experimental coccidian infection would have pronounced effects upon ornamental traits in greenfinches maintained on more natural diets rich in carotenoids and micronutrients remains to be answered in future studies.

LACK OF PROTECTIVE IMMUNITY

Yet another interesting aspect of this study was that drastic effects of experimental infection upon physiology of greenfinches occurred despite the fact that most (89%) of individuals were already infected with isosporan parasites before the experimental treatment. Thus chronic isosporan infection does not appear to provide sufficient protective immunity against subsequent infections with the same parasite. This result may seem surprising, given that avian coccidia are considered highly immunogenic and primarily infections can stimulate solid immunity to homologous challenges (reviewed in Martin *et al*. 1997; Allen & Fetterer 2002b). However, this strong impact of experimental infection in chronically infected greenfinches can be easily reconciled with the concept of antigenic diversity of different strains of the parasite, which means that infection of hosts within one subpopulation of a pathogen fails to induce cross-protective immunity against another. Immunologically relevant genetic diversity within a pathogen population allows different strains of a pathogen to infect hosts that are immune to other antigenic variants and is driven most probably by the acquired immune response of the host population. Under this scenario, host genotype and infection history act in concert to determine the level of cross-protective immunity generated by infection (reviewed by Apanius *et al*. 1997; Smith *et al*. 2002).

Nothing is known about strain specificity of avian *Isospora*, although similarly to those of this study, the results of Brawner *et al*. (2000) and McGraw & Hill (2000) suggest that health impact of experimental infection can be induced in already infected individuals. Notably, recent studies with *E. maxima* in chickens (Smith *et al*. 2002) have demonstrated full protective immunity against the reinfection with the same strain of the parasite, while cross-protection against the heterologous parasite strain varied from zero to almost 100%, depending on host genetics. This is actually the situation predicted by the original Hamilton–Zuk hypothesis of host–parasite arms races, namely that the simultaneous occurrence of genetic polymorphism among both hosts and parasites determines infection success. In fact, coccidia seem to appear ideal parasites for testing the Hamilton–Zuk hypothesis because hosts are likely to acquire continuously multiple infections from diverse strains. Simultaneous coexistence of these different strains within the digestive system of hosts will further expand the antigenic diversity of parasites due to recombination in the process of sexual reproduction within the host. Furthermore, the chronic nature of coccidian infection (which still does not exclude outbreaks of heavy mortality) stresses further the suitability of these parasites as potential causative agents of parasite-mediated sexual selection, because such infections allow males to display and females to benefit from parasite-resistant genes (Hamilton & Zuk 1982). Consistent with this prediction, females have been shown to base their mate choice on ornamental traits affected by coccidian infection in ring-necked pheasants (*Phasianus colchicus*, Hillgarth 1990), wild turkeys (*Meleagris gallopavo*, Buchholz 1995), American goldfinches (Johnson, Dalton & Burley 1993) and house finches (reviewed by Hill, Inouye & Montgomerie 2002).

In conclusion, we have demonstrated drastic effects of infection with *I. lacazei* upon the physiology and expression of carotenoid-based plumage coloration in

greenfinches. We specified that reduced absorption of nutrients (including carotenoids and vitamin E) appeared the plausible main mechanism responsible for these effects. Furthermore, we identified several sensitive haemato-serological markers that can be used for diagnostics of the physiological impact of infection. Our results indicate that the isosporan parasites appear an excellent model system for elucidating the physiological and genetic mechanisms of parasite-mediated sexual selection. However, this study also showed that the pathological effects of coccidian infection do not appear necessarily the single (or even the major cause) for fading of carotenoid-based plumage coloration, which calls for further experiments in this area.

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Supplementary material

The following material is available from: http://www.blackwellpublishing.com/products/journals/suppmat/JAE/JAE870/JAE870sm.htm

Appendix 1. Tail feathers of male greenfinches: their carotenoid content (mg g^{-1}) and colour measurements.

Appendix 2. The repeatabilities of the feather colour measurements.

Appendix 3. Effect of manipulation of parasite load upon condition indices and feather coloration of greenfinches, calculated as mean individual changes (%) compared to pre-experimental state (100%).

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Host resistance and parasite virulence in greenfinch coccidiosis

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Keywords:

Carduelis chloris;
infection resistance;
Isospora lacazei;
multiple infections;
protective immunity;
plasma triglycerides;
virulence.

Abstract

The question why different host individuals within a population differ with respect to infection resistance is of fundamental importance for understanding the mechanisms of parasite-mediated selection. We addressed this question by infecting wild-caught captive male greenfinches with intestinal coccidian parasites originating either from single or multiple hosts. Birds with naturally low pre-experimental infection retained their low infection status also after reinfection with multiple strains, indicating that natural infection intensities confer information about the phenotypic ability of individuals to resist novel strains. Exposure to novel strains did not result in protective immunity against the subsequent infection with the same strains. Infection with multiple strains resulted in greater virulence than single-strain infection, indicating that parasites originating from different host individuals are genetically diverse. Our experiment thus demonstrates the validity of important but rarely tested assumptions of many models of parasite-mediated selection in a wild bird species and its common parasite.

Introduction

Host-parasite relationships have been in the focus of research in evolutionary ecology because parasite-mediated selection has a potential to explain the origin and/or maintenance of sexual reproduction, ornamental traits, and MHC diversity (reviewed in Clayton & Moore, 1997; Little, 2002; Summers *et al.*, 2003). Significant amounts of relevant theory, such as the hypotheses of parasite-mediated sexual selection (Hamilton & Zuk, 1982) and dispersal (Møller & Erritzøe, 2001), and the immunocompetence-handicap hypothesis of Folstad & Karter (1992) have been stimulated and explored in the research of wild animals (particularly birds) and their parasites in natural environments. A crucial assumption of such models is that within a population, the hosts should vary either genetically or phenotypically in resistance to infections while the parasites should vary in virulence. A few experimental tests of this assumption in nondomestic vertebrates originate from studies of fish (e.g. López, 1998; Wegner *et al.*, 2003; Kurtz *et al.*, 2004) and lizards (Oppliger *et al.*, 1999). As regards the birds, two studies of barn swallows *Hirundo rustica* (Møller, 1990; Møller *et al.*, 2004) and a study of kittiwakes *Rissa tridactyla* (Boulinier *et al.*, 1997) have detected significant heritability of ectoparasite resistance. On the other hand, to our knowledge the assumption that parasite strains inhabiting different host individuals may appear genetically diverse has never been experimentally studied in a wild bird species. Assuming that avian models are most likely to remain in the scope of active research of parasite-mediated selection, it would therefore be important to determine the sources of variation in host resistance and parasite virulence in species available for traditional field studies, such as passerine birds.

Among such possible model systems, the association between coccidian intestinal parasites and their avian hosts seems especially promising. Coccidians from the genus *Isospora* (Protozoa, Apicomplexa) infect a number of passerine species (reviewed by Giacomo *et al.*, 1997; Duszynski *et al.*, 2000; McGraw & Hill, 2000). Related coccidians from the genus *Eimeria* are common parasites of poultry where they directly inhibit the uptake of essential dietary components, including carotenoids and other fat-soluble antioxidants, in the gastrointestinal tract of chickens (e.g. Allen & Fetterer, 2002a), and consequently depress carotenoid-based pigmentation ('pale bird syndrome'; Tyczkowski *et al.*, 1991). Thus, in

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the context of the vivid interest of animal ecologists in carotenoid-based ornaments as potential signals of phenotypic quality (e.g. Lozano, 1994; Olson & Owens, 1998; von Schantz *et al.*, 1999; Møller *et al.*, 2000), such parasites should be especially suitable for the detection of mechanisms ensuring the honesty of signals. Indeed, the effect of experimental coccidian infection upon the carotenoid-based ornaments has been detected in three cardueline finch species (Brawner *et al.*, 2000; McGraw & Hill, 2000; Hõrak *et al.*, 2004). Importantly, coccidian infection intensity, measured as concentration of parasite oocysts in faeces, directly indicates parasite reproductive success (e.g. Chapman, 1998). Thus, unlike in many other parasite models, proportional relationships between host resistance, parasite virulence and parasite fitness can be assumed. Another appealing aspect of coccidiosis is that reproduction of parasites can be stopped with coccidiostatic drugs, which enables standardizing the infection status of hosts and later reinfection with parasite strains isolated from different donor individuals.

We address the issues of variation in host resistance and parasite virulence in the study of wild-caught greenfinches and their coccidian parasites. Greenfinches (*Carduelis chloris* L.) are medium-sized (ca. 28 g), sexually dichromatic gregarious seed-eating passerines native to the western Palearctic region. The colour of carotenoid-based feathers has been shown to be a sexually selected trait, as more brightly coloured male greenfinches are favoured by females as mates (Eley, 1991). Our previous study (Hõrak *et al.*, 2004) has demonstrated severe effects of infection with Isosporan coccidians on the physiology and expression of carotenoid-based plumage coloration in greenfinches. This study compared birds inoculated with the mixed parasite strains with those continuously medicated during the experiment. Infection resulted in drastic but transient decreases in serum carotenoid, vitamin E, triglyceride and albumin concentrations, and reduced body mass, indicating serious pathology and probable nutrient malabsorption due to damaged intestinal epithelium. This model system thus proved useful for experimental manipulations of host infection status. In the current study, we use experimental infections and reinfections with homologous and heterologous parasite stocks in order to address the general issue about why individual hosts differ in their parasite loads. Specifically, we ask the following questions:

1. Is the natural variation in parasite loads caused by different resistance of individuals? Animal parasites generally exhibit an aggregated or overdispersed distribution within their host populations (see e.g. Boag *et al.*, 2001 for a review). Such heterogeneities can be generated either by variation between individuals in their exposure to parasites or by differences in their susceptibility to infection (Wilson *et al.*, 2002). These options can be distinguished by infecting hosts with initially low or high infection levels with the same parasite strains. If the differences between natural infection levels are caused by different resistance of those host categories, then the differences in infection intensities between different bird categories should remain prominent also after experimental reinfection with the same parasite strains. Alternatively, if the birds with initially low infection have low parasite loads just because they have not encountered truly virulent pathogens yet, then the new infection should be similarly virulent among the individuals with initially low and high parasite loads.
2. Does encounter with novel parasites confer protective immunity against subsequent infection with the same strains? For the parasite-mediated selection to occur, at least some hosts in the population should remain susceptible to at least some parasite strains present in that population. This means that hosts should not be able to build up effective immunity against any novel parasite strains. We thus predicted that if the same individuals were infected twice with the same parasites, then infection intensity would not decrease after the second infection. Alternatively, if the birds are able to acquire resistance subsequent to each new encounter with a novel strain, then the second infection with the same parasites should result in lower virulence than the first infection.
3. Do heterologous infections (parasites originating from multiple hosts) yield more severe parasitemias than homologous infections with parasites originating from a single host? For the host-parasite coevolution to occur, the parasite strains present in the population must be genetically variable. This assumption can be indirectly tested by comparing infection intensities resulting from heterologous and homologous infections. Genetic variation in parasites inhabiting different host individuals will be manifested if infection with multiple novel strains results in greater virulence than infection with a single novel strain or the host's own parasites. It should be noted, however, that such a result by itself would not be sufficient proof that different parasite strains vary specifically in their virulence. Theoretically, it is also possible that due to competitive host exploitation, multiple infections with strains of similar virulence also leads to higher pathology than infection with the same strains separately (e.g. Wedekind & Rüetschi, 2000; but see Brown *et al.*, 2002). However, in such a case also, those parasite strains cannot be genetically identical.

Materials and methods

A total of 52 male greenfinches were caught in mist-nets in the Sõrve Bird Observatory in Island Saaremaa (57°55′N; 22°03′E) during 2 (day 0) and 3 January 2004. Birds were transported to Tartu and housed in individual indoor cages (27 × 51 × 55 cm) with sand bedding. The birds were fed *ad libitum* with sunflower

seeds and tap water. During the study, birds were kept on the natural day-length cycle. All procedures in the aviary were done in the dark before illumination (hereafter 'morning') or after the lights were turned off (hereafter 'evening'). During the setting of the paper bedding (see *Parasites* section) the lights were turned off. Birds were released on 8 March (day 98). The study was conducted under a license from the Estonian Ministry of the Environment.

Research protocol

The course of the experiment is described in Fig. 1. After transportation to Tartu birds were allowed a 3-day acclimatisation period (days 2–4) in the aviary. After day 5 (days 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, 35 and 38–41) we started the monitoring of individual parasite loads to determine the individual average pre-experimental infection level (hereafter 'pre-exp. oocyst count'). Concurrently (during days 6–26) the oocysts were collected for the experimental inoculations. In the morning of day 39, all the birds were blood sampled (time point 'pre-exp.' in the Fig, 1 and 3–5). In the evening, 2 days later (day 41) all birds were administered a coccidiostatic cure by adding Vetacox PLV (Sanofy-Synthelabo Inc., Paris, France) to their drinking water (1 g of Vetacox dissolved in 2 L of water) for 5 days (days 41–45). During the subsequent 5 days (46–50), the effects of the coccidiostatic treatment waned and a relapse in oocyst counts was detected from the day 48 onward. At the end of this relapse period (day 47) birds were blood sampled for the second time (time point '1. rel.' in the Fig, 1 and 3–5). In the evening of day 51, all birds were inoculated orally with 2000 sporulated oocysts of *Isospora lacazei* diluted in 2×100 μL of water. Birds were allocated to four equal (13 birds) treatment groups, which received different inoculates (however, our sample sizes vary slightly in different analyses due to our inability to measure all variables in all individuals during all the sampling episodes). The first group (hereafter 'own') was inoculated with the oocysts collected from their own faeces while the second (hereafter 'mixture') group was inoculated with a mixture of oocysts collected from six birds with higher than average pre-experimental oocyst counts. The third group (hereafter 'single strain') received oocysts collected from a single bird with the highest oocyst output, and the fourth group (hereafter 'initially low') was inoculated with the same mixture of oocysts as the 'mixture' group. The oocyst mixtures did not contain parasites from the donor of a single strain. The groups did not differ by age ($\chi^2_3 = 0.3$, $P = 0.959$) or body mass ($F_{3,48} = 0.45$, $P = 0.717$). The groups 'own', 'mixture' and 'single strain' did not differ in their pre-experimental oocyst counts ($F_{2,36} = 1.41$, $P = 0.256$), while the 'initially low' group consisted of birds with significantly lower than average pre-experimental oocyst counts ($F_{3,48} = 16.01$, $P < 0.001$).

On the ninth (day 60) morning after the first inoculation, the third set of blood samples was collected (time point '1. inf.' in the Fig, 1 and 3–5) and the same evening, birds entered the second coccidiostatic cure with Vetacox (days 60–64). After the second relapse period (days 65–69), birds were assigned to the second experimental inoculation (day 70). During the second inoculation, birds from the group 'single strain' received oocysts collected from their own faeces and the group 'initially low' received pure tap water (due to shortage of infection material). The birds from groups 'own' and 'mixture' received the same treatment as during the first inoculation. The fourth blood sampling (time point '2. inf.' in the Fig, 1 and 3–5) was performed on the ninth morning after the second inoculation (day 79). On the 86th day of the experiment, all the birds were injected intradermally in the wing web with 0.2 mg of phytohaemagglutinin (PHA) in 0.04 mL of isotonic saline in order to measure cell-mediated immunity. On day 88, all the birds were injected with a 50 μL suspension of sheep red blood cells (SRBC) diluted in sterile isotonic saline to induce the humoral immune response. Results of these experiments will be reported elsewhere.

Plasma triglyceride concentrations from each blood sampling were determined by enzymatic colorimetric test

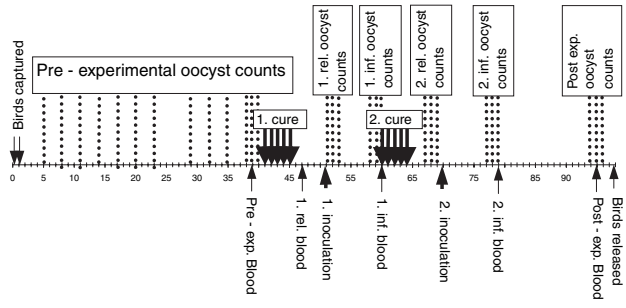

Fig. 1 Course of the experiment. Day 0 = 2nd January. Boxes 1. cure and 2. cure indicate the days of administration of the coccidiostatic treatment. Boxes describing oocyst counts indicate the periods over which the daily oocyst counts (dotted lines) were averaged. 1. and 2. inf. stand for the first and second experimental infection, respectively. 1. and 2. rel. denote measurements of infection intensities during the periods of natural relapses of infection, subsequent to the periods of medication with a coccidiostatic drug.

as described in Hõrak *et al.* (2004). High blood triglyceride levels are indicative of a resorptive state during which fat is deposited to adipose tissues. Hence triglyceride concentrations reflect the individual's state of fattening by indicating the amount of food absorbed during the few hours before blood sampling (Jenni-Eiermann & Jenni, 1998). To assess the intensity of coccidian infections, faecal samples were collected during 3 days around the blood samplings and the averages of parasite counts for these 3 days were used in statistical analyses (days 51–53 for point '1. rel.', days 58–60 for paint '1. inf.', days 67–69 for point '2. rel.', days 77–79 for point '2. inf.' and days 94–96 for points 'post-exp.' in the Fig, 1 and 3–5).

Parasites

The coccidian species present in the faeces of migrating greenfinches in Estonia has been previously identified as *I. lacazei* (see Hõrak *et al.*, 2004 for details). Since coccidian parasites are known to be highly host specific (e.g. Lillehoj & Trout, 1993) it was assumed that birds used in the current data set were infected with the same species of *Isospora*.

Because of diel periodicity in oocyst shedding (e.g. Brown *et al.*, 2001), two sheets of paper (paper bedding) were placed upon the sand bedding in the individual birdcages 2 h before turning off the lights. After the lights were turned off in the evening, the faeces were collected from the papers. Faecal samples were weighed to the nearest 0.01 g with an electronic balance (Mettler Toledo AB-S), suspended in 1 mL of water and held at room temperature for 30 min. Then, the solution was drained through gauze into individual tubes and centrifuged at 1500 r.p.m. (179 ***g***) for 7 min. The supernatant was removed and 0.5 mL of saturated NaCl water solution was added to the 0.5 mL of residue. The number of oocysts was counted using the McMaster chamber (volume = 0.15 mL) and their concentration was expressed as number of oocysts per gram of faecal sample. Repeatability of infection intensity, measured from two faecal samples collected at the same time, was 0.91 ($F = 20.34$; $P < 0.0001$; $n = 20$). During the pre-experimental period, coccidiosis was diagnosed for all the birds with an average intensity of 105918 ± 354320 (SD) oocysts per g. Difference in individual infection intensities was very high, ranging from 266 ± 552 to 2502444 ± 1415898 oocysts per g (however, the second highest infection intensity was already considerably lower than the maximal, with an average pre-experimental oocyst count of 487638 ± 804029 oocysts per g). The distribution of the pre-experimental parasite loads was highly aggregated (Fig. 2).

Oocysts to be used for oral inoculations were collected during the 20-day period before the first blood sampling (days 6–26). Faecal samples of each bird were pooled to individual cell culture flasks with 75 cm^2 culture area and filter caps for continuous venting, and preserved in 2% potassium dichromate ($K_2Cr_2O_7$) solution at room temperature and aerated daily. Sporulation of oocysts was registered 15 days after collecting the last sample (day 41) by microscopic observation. To prepare the inoculates, the mixture was drained through gauze and the resulting potassium dichromate solution containing oocysts centrifuged at 2500 r.p.m. (496 ***g***) for 10 min. After centrifugation, the supernatant was removed and 0.2 mL of residue was resuspended in 1 mL of water. This mixture was centrifuged again at 2500 r.p.m. (496 ***g***) for 10 min and the supernatant removed leaving 0.2 mL of residue. This washing procedure was

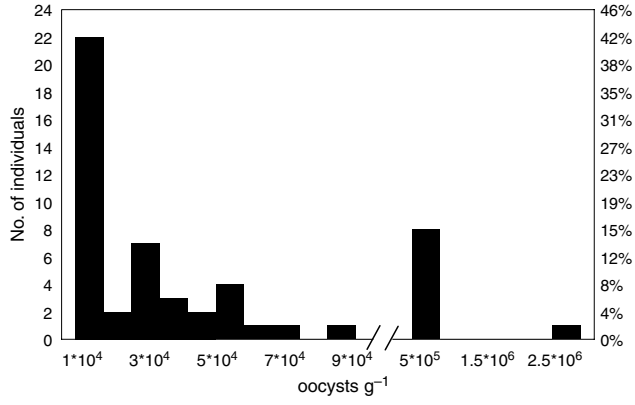

Fig. 2 Frequency distribution of average pre-experimental infection intensities.

repeated 3–4 times until the potassium dichromate was removed from the solution.

Results

Infection dynamics: group averages

During the 13 sampling days of the pre-experimental period, infection intensities of individual birds were moderately but significantly repeatable ($r = 0.43$, $F_{51,727} = 12.11$, $P < 0.00001$). After the first experimental infection, birds inoculated with the multiple strains developed higher infection intensity than birds inoculated with their own strain (Fig. 3a; $F_{5,120} = 3.91$, $P < 0.01$ for time × group interaction term in repeated measures ANOVA with main effects of group ($F_{1,24} = 0.04$, $P = 0.837$) and time ($F_{5,120} = 2.39$, $P < 0.05$)). Average infection intensity in the former group also remained higher than that of the birds inoculated with their own strain during the periods subsequent to the second medication and second infection. Birds infected with the single external strain developed infection

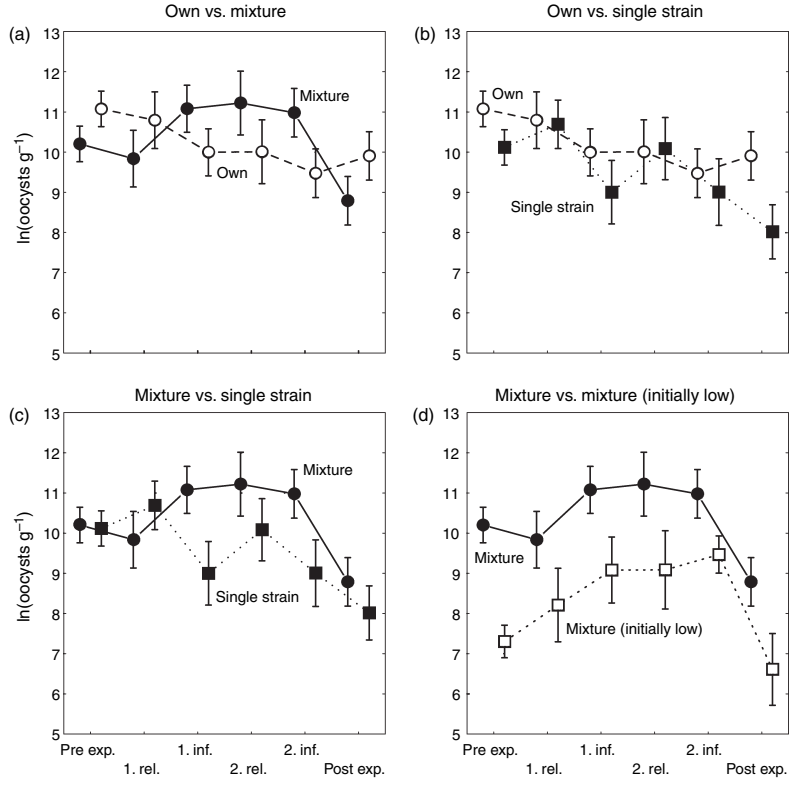

Fig. 3 Effect of experimental infections upon the coccidian oocyst shedding (per gram of feces) in different treatment groups. 'Own' stands for double infection with own strain; 'mixture' denotes double infection with mixture of strains in 'susceptible' hosts; 'single strain' is for infection with a single external strain (second time infected with own strain) and 'mixture (initially low)' denotes infection with a mixture of strains in 'initially low' hosts (second time treated with water). Exact time intervals for sampling are shown in Fig. 1. Coccidian reproduction was completely arrested both before the first and second infection (not shown in the figure), $n = 12$–13 birds per group. Vertical bars are SE. In repeated measures ANOVA, including all time points depicted on the figure, time × group interaction term is statistically significant ($F_{15,230} = 2.44$, $P = 0.003$) when all groups are included in a single model with main effects of group ($F_{3,46} = 2.83$, $P = 0.049$) and time ($F_{5,230} = 6.17$, $P < 0.001$).

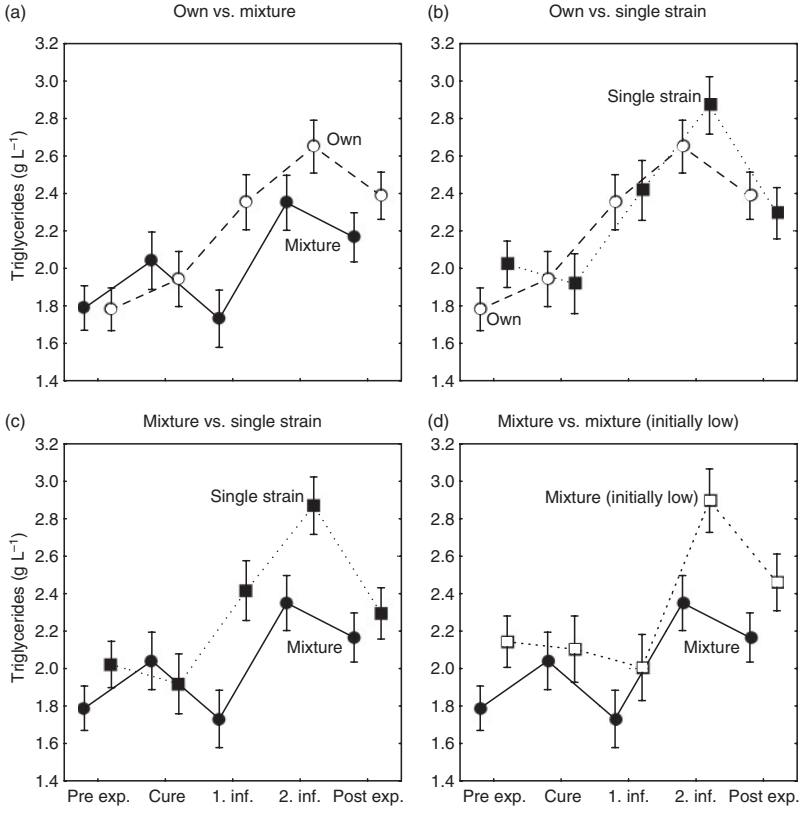

Fig. 4 Effect of experimental infections upon the plasma triglyceride concentration in different treatment groups. See the legend of Fig. 3 for the details. Time × group interaction term is statistically significant ($F_{12,164} = 2.78$, $P = 0.002$) when all groups are included in a single model with main effects of group ($F_{3,41} = 1.60$, $P = 0.204$) and time ($F_{4,164} = 32.83$, $P < 0.001$).

dynamics, indistinguishable from the birds infected with their own parasites (Fig. 3b; $F_{5,115} = 1.46$, $P = 0.209$ for time × group interaction in the model with main effects of group ($F_{1,23} = 0.81$, $P = 0.376$) and time ($F_{5,115} = 6.06$, $P < 0.001$)). Subsequent to the first infection, birds infected with the single external strain also developed weaker infection than birds infected with multiple strains (Fig. 3c; $F_{5,115} = 3.28$, $P < 0.01$ for time × group interaction term in the model with main effects of group ($F_{1,23} = 0.04$, $P = 0.837$) and time ($F_{5,115} = 2.39$, $P < 0.05$)). Comparison of birds with relatively low and high average pre-experimental infection intensity (but infected with the same multiple strains) revealed that infection dynamics was parallel in time in both groups (Fig. 3d; $F_{5,115} = 0.36$, $P = 0.877$ for time × group interaction term in the model with main effects of group ($F_{1,23} = 7.09$, $P < 0.05$) and time ($F_{5,115} = 6.00$, $P < 0.001$)). The significant main effect for the group factor indicates that both groups remained different in their average infection intensities throughout the experiment.

Plasma triglyceride concentrations followed similar pattern as infection dynamics. Triglyceride levels of birds inoculated with the multiple strains dropped sharply after the first infection and remained generally lower than those of birds infected with their own strain (Fig. 4a; $F_{4,92} = 4.36$, $P < 0.01$ for time × group interaction term in the model with main effects of group ($F_{1,23} = 2.66$, $P = 0.117$) and time ($F_{4,92} = 16.57$, $P < 0.001$)). The same holds for the comparison of birds

inoculated with mixture vs. single external strain (Fig. 4c; $F_{4,84} = 4.63$, $P < 0.01$ for time × group interaction term in the model with main effects of group ($F_{1,21} = 2.95$, $P = 0.101$) and time ($F_{4,84} = 14.17$, $P < 0.001$)). Again, the birds infected with the single strain and their own parasites remained indistinguishable in their triglyceride levels (Fig. 4b; $F_{4,88} = 0.94$, $P = 0.419$ for time × group interaction term in the model with main effects of group ($F_{1,22} = 0.25$, $P = 0.626$) and time ($F_{4,88} = 22.2$, $P < 0.001$)). With regard to the comparison of two bird categories infected with the mixed strains, birds with initially low infection intensity had relatively higher plasma triglyceride levels before the experiment and after the second infection. However, the group factor in the model was only marginally significant ($F_{1,19} = 4.21$, $P = 0.054$) in a model with effects of time ($F_{4,76} = 16.89$, $P < 0.001$) and time × group interaction term ($F_{4,76} = 1.38$, $P = 0.259$; Fig. 4d).

Body mass dynamics during the experiment were generally parallel to that of triglycerides (Fig. 5). However, in this case no significant interactions were found when comparing a group with multi-strain infection with those infected with their own strain (Fig. 5a; $F_{4,96} = 1.08$, $P = 0.354$ for time × group interaction term in the model with main effects of group ($F_{1,24} = 1.38$, $P = 0.252$) and time ($F_{4,96} = 26.5$,

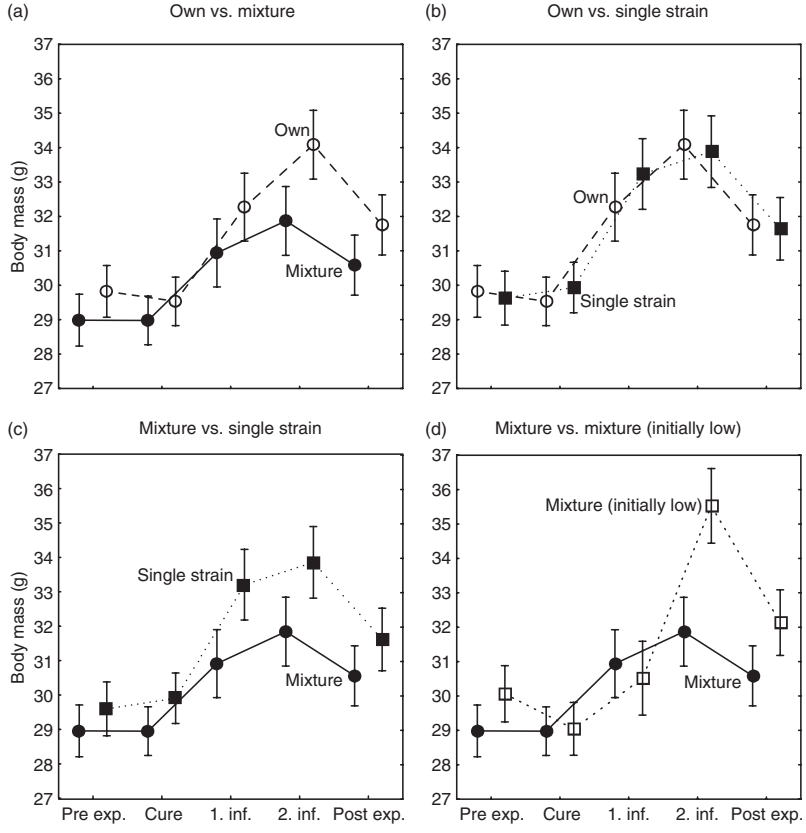

Fig. 5 Effect of experimental infections upon the body mass dynamics in different treatment groups. See the legend of Fig. 3 for the details. Time × group interaction term is statistically significant ($F_{12,180} = 2.36$, $P = 0.008$) when all groups are included in a single model with main effects of group ($F_{3,45} = 0.71$, $P = 0.549$) and time ($F_{4,180} = 50.09$, $P < 0.001$).

$P < 0.001$)). The same holds for the comparison of mixture vs. single external strain (Fig. 5c; $F_{4,92} = 0.98$, $P = 0.385$ for time × group interaction term in the model with main effects of group ($F_{1,24} = 1.54$, $P = 0.227$) and time ($F_{4,92} = 18.86$, $P < 0.001$)). Mass dynamics of birds inoculated with own parasites and single strain were virtually identical (Fig. 5b; $F_{4,92} = 0.42$, $P = 0.655$ for time × group interaction term in the model with main effects of group ($F_{1,23} = 0.03$, $P = 0.860$) and time ($F_{4,92} = 22.48$, $P < 0.001$)). However, this time a different pattern emerged in the comparison of groups with initially high and low infection intensities and infected with the same mixture strain. Subsequent to the second infection (when the former group received second time the same mixture and the latter group received water), body mass of the water-treated 'initially low' group rose significantly higher than that of the infected group. This was also reflected in significant time × group interaction term in the model ($F_{4,88} = 6.01$, $P < 0.01$) with main effects of group ($F_{1,22} = 0.94$, $P = 0.344$) and time ($F_{4,88} = 32.22$, $P < 0.001$; Fig. 5d).

Infection dynamics: individual patterns

To describe the individual patterns of susceptibility to infection, we introduce the term 'response to infection' to denote a difference in individual infection intensities measured during the peak phase of the first infection (data point 3 in Fig. 3) and during the whole pre-experimental period. In 50% of birds, infection intensity increased while in the other half of the birds, infection intensity declined subsequent to the first infection. Birds with different responses to infection were not equally distributed between treatment categories ($\chi^2_3 = 8.3$, $P < 0.05$). Among both groups inoculated with mixed parasite strains, 9 birds of 13 (69%) increased their infection intensities, while among the birds inoculated with their own strain, only 3 of 13 (23%) increased in their infection intensities. This difference between groups was significant ($P < 0.05$, Fisher exact test). Response to infection was intermediate among the birds inoculated with a single external strain (5 birds of 13, i.e. 38% increased in infection intensities). This proportion did not differ significantly from that observed among birds infected with their own strain or among birds infected with multiple strains ($P > 0.2$).

Birds whose infection intensity increased after the first infection evidently suffered deterioration of their physiological condition as the response to infection correlated negatively with the change in plasma triglyceride concentration between first infection and pre-experimental period ($r = -0.34$, $P < 0.05$, $n = 50$). Change in plasma triglyceride levels, in turn, correlated strongly with the corresponding change in body mass during the same period ($r = 0.79$, $P < 0.0001$, $n = 50$).

Discussion

Typical for most animal parasites, the distribution of infection intensities among greenfinches was highly aggregated (Fig. 2). Our experiment succeeded in generating different patterns in infection dynamics among greenfinches infected with coccidian oocysts originating from different hosts. The study also confirmed our assumption that host resistance varies proportionally with parasite virulence (i.e. damage caused to the host) and parasite fitness (i.e. its reproductive rate). This was indicated by the patterns in plasma triglyceride levels and body mass dynamics in different treatment groups, which were generally inversely proportional to the patterns of oocyst output in the same time periods. Furthermore, individual changes in plasma triglyceride levels; body mass and infection intensities were significantly intercorrelated. These results mean that our experimental inoculations caused significant changes in physiology of treated birds. Application of these premises implies that our model system is suitable for explorations of the sources of variation in host resistance and parasite virulence. However, it should also be noticed that not all the changes in host physiology that occurred during our study were due to experimental infections. For instance, transient increases of body mass and plasma triglyceride levels after first infection might have occurred due to habituation of birds to captivity and/or handling stress or changes in hormonal profiles due to increase in day length. Similarly, the decline in body mass and triglyceride levels at the end of the experiment could probably be related to stress and/or extra energetic expenditures associated with immune responses to PHA and SRBC. It is therefore important to rely on between-group differences in infection dynamics (i.e. time × treatment interaction terms in repeated measures ANOVA models) when interpreting the results of our experiment. Next, we will discuss our main findings in the light of questions concerning sources of variation in parasite virulence and host resistance as posed in the Introduction.

First we asked whether the natural variation in parasite loads is caused by differences in resistance of birds to standard infection. This hypothesis was most clearly supported by the result that infection intensities of birds with initially low parasitemia remained low throughout the experiment, although they received exactly the same heterologous inoculum as the birds with average pre-experimental infection intensity. The infection dynamics of these two groups remained parallel in time, although consistently lower among 'initially low-infection' birds (Fig. 3d). This result implies that natural infection intensities confer information about the ability of individuals to also resist novel strains. This is an important finding in the context of immunoecological research where the relative importance of different sources of variation in natural infection levels has been

under continuous debate (e.g. Clayton, 1991; McLennan & Brooks, 1991; Poulin & Vickery, 1993; John, 1997). Furthermore, intra-populational variation in host resistance, especially when occurring simultaneously with variation in parasite virulence, is an important assumption of models of parasite-mediated selection listed in the Introduction. As regards the coccidian infection, various breeds of chickens have been shown to differ remarkably in their resistance to challenge with standard Eimerian strains (e.g. Pinard-van der Laan *et al.*, 1998; Smith *et al.*, 2002). However, to our knowledge, the results of the present study appear to be the first experimental demonstration of individual variation in resistance to coccidiosis in a wild animal population.

At present, we cannot distinguish whether between-individual differences in susceptibility to coccidiosis were primarily caused by genetic or ontogenetic differences in immune function of individual greenfinches. Maternal effects (e.g. Grindstaff *et al.*, 2003) and environmental conditions experienced during ontogeny (e.g. Blount *et al.*, 2003) have been shown to exert considerable effects on individuals' capability to respond to immune challenges later in life. On the other hand, in domestic chickens the outcome of coccidian infection has been shown to depend on the interactions between the genes of both host and parasite. A recent study of *E. maxima* infection in chickens (Smith *et al.*, 2002) has demonstrated full protective immunity against the reinfection with the same strain of the parasite, while cross-protection against heterologous parasite strain varied from zero to almost 100%, depending on host genetics. Yet it is at present unknown whether genetic variation in parasite resistance is also responsible for the differential susceptibility to coccidian infections in wild birds. The similarity of the natural situation to the above-mentioned one is not necessarily obvious because of the vastly different selection pressures in the wild as compared to those imposed by the past and current poultry industry (e.g. Knap & Bishop, 2000). However, as regards greenfinches, any mechanism leading to consistent between-individual differences in parasite resistance would be sufficient for triggering parasite-mediated sexual selection. This is because in a species where females can gain direct benefits by mating with resistant males, it does not matter whether individual differences in disease susceptibility are of genetic or environmental origin. In greenfinches the resistance to coccidiosis is likely to affect the quality of the parental care provided by males, as suggested by the serious health impact of infection, detected in this study and by Hõrak *et al.* (2004). Second, birds whose general condition is weakened by coccidiosis might be more susceptible to infections transmitted via physical contact and thus more likely to infect their breeding partners. Third, infected males with a weakened condition might be more vulnerable to predation (e.g. Møller & Erritzøe, 2000) during breeding, which would again put a premium on females to mate with more resistant individuals. Coccidian infection has been shown to depress the expression of carotenoid-based plumage coloration in greenfinches (Hõrak *et al.*, 2004) and those ornaments are targets of female choice (Eley, 1991). It is thus likely that proceeding from the indicators of resistance to coccidiosis in their mate choice would enable greenfinch females to obtain at least direct benefits from their mates.

Our second question was whether the encounter with novel parasites confers protective immunity against subsequent infection with the same strains. This is an important issue because parasite-mediated selection could not work if hosts were able to build up effective immunity against any novel parasite strains. In our study, average infection intensity did not decrease after secondary infection among birds that were repeatedly infected with the same heterologous parasites. This result indicates that encounters with a mixture of parasite strains did not help the birds to suppress efficiently the subsequent infection with the same strains. This finding also supports our previous contention that individuals really differed in their general capability to resist coccidiosis. Because we could not observe the development of protective immunity at reinfection with the heterologous inoculum, we can eliminate the possibility that birds with initially low parasite loads retained their low infection status during the experiment just because they were already familiar with the strains contained in that mixture. Although, to our knowledge, the development of immunity against avian *Isospora* has never been described, we believe that the time interval between subsequent infections (19 days) was sufficient to enable birds to develop the immunological memory. According to previous work on chicken coccidiosis, birds can develop immunity against homologous strains during 2–4 weeks after initial inoculation (e.g. Vermulen *et al.*, 2001) and parasite reactive serum antibodies reach maximum levels at 8–14 days after oral infections (Lillehoj & Ruff, 1987). We thus consider it likely that the lack of protective immunity due to previous exposure to the same parasite strains reflects the genuine inability of greenfinches to become coadapted with just any coccidian strains encountered during their lifetime. On the other hand, the result that reinfection of hosts with their own parasite fauna resulted in lower infection intensities than infection with mixed strains (Fig. 3a) indicates that hosts can tolerate their 'own', previously acquired parasites better than novel ones. Such a situation can occur, for instance, if each observed individual represents a viable and unique host-parasite assemblage, retained after selective elimination of such host-parasite combinations, which resulted in deadly overexploitation of hosts. Under such a scenario, the outcome of infection would depend on the genetic variation between both hosts and parasites as predicted by the Red Queen models of host-parasite coevolution (e.g. Frank, 1994). Such a scenario seems plausible, given

that chickens can acquire immunity against Eimerian infections on the basis of MHC-mediated responses, although, notably, the innate immune responses also play an important protective role (Allen & Fetterer, 2002b).

The question whether coccidian strains inhabiting greenfinches are genetically heterogeneous was addressed by comparing the infection success of parasite inocula originating from single or multiple hosts. We expected that genetic variation among parasite strains infecting different hosts would be revealed by the higher virulence of parasites originating from multiple hosts as compared to infection with parasites originating from a single host. Consistent with our expectations, birds inoculated with multiple strains developed higher infection intensities than birds infected with parasites from a single host (Fig. 3c). This also means that our assumption about different individuals harbouring different *Isosporan* strains was justified. This was expected on the basis of previous research on chicken coccidiosis where different strains of coccidia are known to interbreed within a host (e.g. Williams, 2002) which means that infection material originating from a single individual can be considered relatively homologous. Thus, although we do not know how many different founder strains participate in the forming of the coccidian fauna of individual greenfinches, our results suggest that between individual differences resulting in individual faunas are sufficient to lead to differential pathogeneicity. However, the patterns of multiple infections resulting in greater virulence than infection with a single novel strain or a host's own parasites can have at least four possible explanations.

First, and probably the most parsimonious explanation would be that because parasite strains differ in their virulence, hosts are more likely to encounter virulent parasites from multiple rather than from single infections. Such an outcome would be compatible with previous findings about immunological variability of different Eimerian strains in chicken coccidiosis (e.g. Martin *et al.*, 1997; Williams, 1998, 2002; Smith *et al.*, 2002) and microparasite infections in general (reviewed by Read & Taylor, 2001). It is noteworthy in this context that infection with a single novel strain could even be considered avirulent in our study because such birds had infection dynamics (Fig. 3b), as well as patterns in plasma triglyceride levels (Fig. 4b) and body mass (Fig. 5b) which were virtually indistinguishable from the birds of the control group infected with their own parasites. Given that coccidians invading previously infected host are expected to face fierce competition by preceding strains (e.g. Williams, 1998), and more virulent parasite strains are generally supposed to outcompete milder strains (reviewed in Wedekind, 1999), it seems plausible that coccidians from a single host were less likely to compete with pre-existing strains than coccidians originating from multiple hosts.

Second, a slightly modified version of the first explanation would be that multiple infections increase the likelihood of encounter between such host and parasite genotypes that result in the most virulent infections. Such an outcome would be expected under many models of host-parasite evolutionary dynamics (e.g. Frank, 1994) and it would also compare favourably with findings of genotypic interactions between host and parasites in chicken coccidiosis described above.

The third possibility is that multiple infections lead to higher parasite replication for mechanistic reasons because simultaneously fighting antigenic variants of different parasite strains might be more difficult for the host's immune system than handling a single-strain infection. Such an explanation would be compatible, for instance, with the findings of de Roode *et al.* (2003) who showed that mixed clone infections were harder to clear than single clone infections in the murine malaria.

Finally, at least theoretically, we cannot exclude the scenario where parasite strains inhabiting different hosts possess similar virulence but different degrees of relatedness to each other. This explanation would be based on the reasoning that the optimal rate of host exploitation, and hence virulence, is higher in genetically diverse infections because in-host relatedness is reduced (e.g. Wedekind & Rüetschi, 2000; Read & Taylor, 2001; but see Brown *et al.*, 2002). However, even such a scenario would require that parasite strains inhabiting different host individuals are genetically diverse.

To summarize, the results of our experiment indicate that the outcome of coccidian infection in greenfinches depends on concurrent variation in host resistance, parasite virulence and their interaction. Importantly, we showed that natural infection intensities reflect individuals' ability to resist novel strains and that greenfinches do not develop protective immunity against arbitrary parasite strains. This study also showed for the first time in a wild bird species that coccidian parasites inhabiting different host individuals are genetically diverse, which demonstrates the validity of important but rarely tested assumption of many models of parasite-mediated selection. In line with our findings, Bensch & Åkesson (2003) have demonstrated spatial and temporal variation in different lineages of *Haemoproteus* sp. blood parasites in Swedish willow warblers (*Phylloscopus trochilus*). Similarly, temporal variation in different lineages of an avian malarial parasite community in Puerto Rico has been detected by Fallon *et al.* (2004). However, not much is known about the fitness consequences of avian malaria infections, and therefore it is unclear whether they will contribute to the maintenance of variation in the host's resistance genes. We consider it much more likely that Isosporan coccidians can play such a role in passerine birds, because their pathogeneicity (which can ultimately lead to the host's death) has been well documented (Box, 1977; Sironi, 1994; Giacomo *et al.*, 1997; Hõrak *et al.*, 2004). This suggests that avian coccidiosis offers a great

potential for microevolutionary research, especially in the context of current advances in molecular characterisation of different parasite strains and host immune system diversity.

Acknowledgments

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Do standard measures of immunocompetence reflect parasite resistance? The case of Greenfinch coccidiosis

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Summary

1. Much research in evolutionary animal ecology is currently focused on issues related to parasite-mediated selection. Because of difficulties in estimation of actual parasite resistance, researchers often rely on surrogate measures of immunocompetence, such as the magnitude of immune responses to artificial antigens, assuming that these measures correlate with individuals' ability to resist disease.
2. This assumption was tested in the Greenfinch (*Carduelis chloris* L.) coccidiosis model by measuring the association between parasite resistance and two standard assays of immune function – a swelling response to phytohaemagglutinin (PHA) and antibody production against sheep red blood cells (SRBC).
3. Before performing immune challenge assays, host resistance to the intestinal parasite *Isospora lacazei* (Labbe) was assessed on the basis of individual infection intensities and responses to challenge infection with alien parasite strains.
4. Increases in infection intensities after inoculation of birds with novel parasite strains correlated positively with magnitude of swelling response to PHA injection. Thus, intense parasite multiplication in the host's digestive tract resulted in the enhancement of the host's cell-mediated immune function.
5. Among birds that were vulnerable to novel infections, no correlations emerged between infection intensities, SRBC antibody titres and condition indices.
6. Among non-vulnerable hosts (whose infection intensities did not increase after experimental reinfection), SRBC antibody titres correlated negatively with infection intensities and positively with indices of nutritional condition. Hence, high SRBC antibody titres reflected good nutritional status and superior resistance to an important parasite species in a subsample of studied Greenfinches.
7. Altogether, our results exemplify the diversity of outcomes when the host's immune system is simultaneously challenged by natural enemies and artificial antigens, and call for the cautious interpretation of the results of standard immune tests in the context of parasite resistance.

Key-words: *Carduelis chloris*, *Isospora lacazei*, phytohaemagglutinin, plasma triglycerides, SRBC

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Introduction

Parasite-mediated selection is currently believed to play an important role in the evolution of life histories and ornamental traits (reviewed in Clayton & Moore 1997; Zuk & Stoehr 2002) and the regulation of population dynamics (reviewed in Hudson *et al*. 2002). Research on these areas therefore often requires estimation of individuals' parasite resistance. The latter, however, has often turned out to be a non-trivial task, and the measurement of host antiparasite defences rather than parasite intensities has therefore been recommended (e.g. Møller, Christe & Lux 1999). In recent decades, the amount of research on the ecological and evolutionary sources of variation in the immune function of different organisms has been growing (Sheldon & Verhulst 1996; Zuk & Stoehr 2002), with much focus on the search for the most objective and easily applicable methods for measuring the immune function in the field (e.g. Smits & Baos 2004; Møller & Saino 2004). Among such assays, measurements of immune responses elicited by inoculations with various foreign antigens are gaining increasing popularity, because such assays are believed to estimate the general potential of the immune system to respond to the novel challenge (e.g. Faivre *et al*. 2003; Smits & Baos 2004; Møller & Saino 2004). The implicit assumption

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of such tests, namely that immunoresponsiveness correlates with the ability to resist disease, or at least indicates the relative 'strength' of the immune system, however, has been challenged (Owens & Wilson 1999; Hanley & Stamps 2002; Ryder 2003; Adamo 2004). The questions of whether and how the measurements of 'immunocompetence' obtained from standard immune challenge protocols reflect real parasite resistance therefore require clarification. In this study, we ask how parasite resistance associates with standard measures of immunocompetence in experimental coccidian infection in Greenfinches.

Coccidians from the genus *Isospora* (Protozoa, Apicomplexa) are directly transmitted intestinal parasites that infect a number of passerine species (reviewed by Giacomo *et al.* 1997; Duszynski, Couch & Upton 2000; McGraw & Hill 2000). Greenfinches are medium-sized (*c.* 28 g), sexually dichromatic gregarious seed-eating passerines native to the western Palearctic region. Our previous study (Hõrak *et al.* 2004) has demonstrated severe effects of infection with Isosporan coccidians on the health state and expression of carotenoid-based plumage coloration in Greenfinches.

In order to test whether individuals' ability to mount immune responses against artificial antigens reflects their resistance to coccidiosis, we injected the birds with phytohaemagglutinin (to measure the general potential for cell-mediated immune response) and sheep red blood cells (SRBC) to measure the ability for antibody production. These two assays are often used as the estimates of immunocompetence (reviewed in Zuk & Stoehr 2002; Smits & Baos 2004), which, in turn, is believed to be ultimately linked to fitness via general parasite resistance (e.g. Gustafsson *et al.* 1994; Møller *et al.* 1999). However, the validity of the latter link has seldom been tested in wild birds (Owens & Wilson 1999; Tella, Scheuerlein & Ricklefs 2002; Ryder 2003). According to the hypothesis that immune responses to artificial antigens reflect infection resistance, we predicted negative correlations between SRBC antibody titre and PHA response *vs* infection intensities, and positive correlations between measures of immunocompetence and individuals' ability to resist novel parasite strains. Additionally, we tested whether immune responsiveness correlates with individuals' nutritional status by measuring correlations between plasma triglyceride concentrations, body mass and magnitude of immune responses. Because parasitism and immune responses are condition dependent, we also ask whether those relationships differ between individuals with different degrees of vulnerability to novel infections.

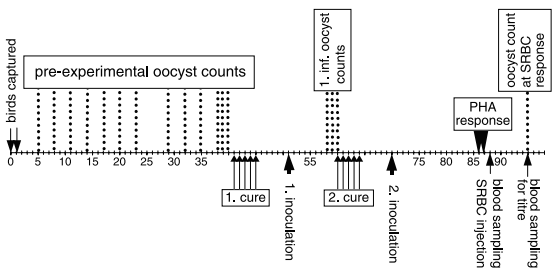

Fig. 1. The course of the experiment. Day 0 = 2 January. Boxes '1. cure' and '2. cure' indicate the days of administration of the coccidiostatic medication. Boxes describing oocyst counts indicate the periods over which the daily oocyst counts (dotted lines) used in this study were averaged. 1 and 2. inf. stand for the first and second experimental inoculation, respectively.

Materials and methods

Male greenfinches were caught in mist-nets in the Sõrve Bird Observatory on the island of Saaremaa (57°55′ N, 22°03′ E) during 2 (day 0) and 3 January 2004. Birds were transported to Tartu and housed in individual indoor cages ($27 \times 51 \times 55$ cm) with sand bedding. The birds were fed *ad libitum* with sunflower seeds and tap water. During the study, birds were kept on the natural day-length cycle. All procedures in the aviary were carried out in the dark before illumination (hereafter 'morning') or after the lights were switched off (hereafter 'evening'). During the procedures for testing individual cellular immune response (performed during mid-day, from 12.00 to 14.20) and setting the paper bedding (see *Parasites* section) the lights were turned off. Birds were released on 8 March (day 98). The study was conducted under licence from the Estonian Ministry of the Environment.

RESEARCH PROTOCOL

The course of the experiment is shown in Fig. 1. After transportation to Tartu, birds were allowed a 3-day acclimatization period (days 2–4) in the aviary. After day 5 we started monitoring the individual parasite loads (days 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, 35, 38, 39, 40, 41) to determine the individual average pre-experimental infection level. Concurrently (during days 6–26) the oocysts were collected for the experimental inoculations. In the evening of day 41, all birds were assigned to coccidiostatic medication by adding Vetacox PLV (Sanofy-Synthelabo Inc., Paris, France) to their drinking water (1 g of Vetacox dissolved in 2 l water) for 5 days (days 41–45) to standardize their pre-experimental condition. During the subsequent 5 days (46–50), the effects of coccidiostatic medication waned, and relapse in oocyst counts was detected from day 48 onward. (Vetacox is a sulphonamide drug that only stops the replication of coccidians, but does not clear the parasites from the birds. This is evidently the reason why the birds most intensely infected before medication will also be the most heavily infected after the treatment. As reported by Hõrak *et al.* (2004), Greenfinches kept in captivity without anticoccidial treatment may become as intensively infected as the birds experimentally infected with 2000 oocysts.) In

the evening of day 51, all birds were inoculated orally with 2000 sporulated oocysts of *Isospora lacazei* diluted in 2×100 µl water. A total of 26 birds were inoculated with a mixture of oocysts collected from six birds with higher than average pre-experimental oocyst counts. The remaining 13 birds received a homologous inoculum of oocysts collected from a single bird with the highest oocyst output. Results of this part of the experiment, aimed at explaining the variation of the virulence of repeated single *vs* mixed-strain infections, are reported by Hõrak *et al*. (2006).

On the ninth evening (day 60) after the first inoculation, birds were administered a second medication with Vetacox (days 60–64). After the second relapse period (days 65–69), birds were assigned to the second experimental inoculation on day 70. A total of 13 of the birds previously infected with the mixed inoculum received the same inoculum, while another 13 birds from the same group received water. Birds previously infected with a homologous inoculum received oocysts collected from their own faeces.

Cell-mediated immune responsiveness of birds was estimated on the basis of the PHA injection assay, following the simplified protocol of Smits, Bortolotti & Tella (1999) as described in detail by Saks, Ots & Hõrak (2003). The measurement error was reasonably low, since the repeatability (Lessells & Boag 1987), based on three consecutive measurements, was 0·87 ($F = 20{\cdot}85$; $P < 0{\cdot}0001$; $N = 49$). This procedure was performed on days 86 and 87. On the following morning (day 88) all the birds were injected with a suspension of sheep red blood cells (SRBC) diluted in sterile isotonic saline to induce the humoral immune response as described by Saks *et al*. (2003). Blood samples for assessment of SRBC response and plasma triglyceride concentrations were obtained 8 days after the injection (day 95). SRBC antibody titres were measured using a haemagglutination assay (Wegmann & Smithies 1966; Lawler & Redig 1984) as described in detail by Saks *et al*. (2003). Plasma triglyceride concentrations were determined by enzymatic colorimetric test as described in Hõrak *et al*. (2004). High blood triglyceride levels are indicative of a resorptive state during which fat is deposited to adipose tissues. Hence triglyceride concentrations reflect the individual's state of fattening by indicating the amount of lipids absorbed during the few hours before blood sampling (Jenni-Eiermann & Jenni 1998). Blood for triglyceride measurements was collected after nocturnal fast, when the birds were in postresorptive state. Hence, between-individual variation in plasma triglyceride levels in our study reflects the variation in basic nutritional state, independently of recent food intake.

To assess the intensity of coccidian infections, faecal samples were systematically collected during the experiment. In the analyses we use individual parasite counts measured (1) during the whole pre-experimental period (average of 15 samplings during the period from the 5th to 41st day of the study, (2) during 3 days (averaged over days 58–60) subsequent to the first experimental infection and (3) during the evening of measuring SRBC antibody titres on day 95 (Fig. 1). The rationale of using different parameters to characterize the infection was based on the concept that different aspects of parasite resistance are controlled by different properties of the immune system. The average pre-experimental infection intensity reflects an individual's ability to suppress chronic infection. However, this ability does not necessarily reflect the individual's ability to cope with novel antigenic variants of the parasite. To measure the latter, we introduce the term 'vulnerability to novel infections'. This was quantified as the difference in individual infection intensities measured during the peak phase of first experimental infection and during the whole pre-experimental period. We are confident that infections with coccidian strains of unfamiliar origin indeed affected the Greenfinches in our study, as revealed by the significant effects of experimental inoculation on infection intensity, body mass and plasma triglyceride levels (Hõrak *et al*. 2006).

PARASITES

Coccidia of the genus *Isospora* are obligate intracellular parasitic protozoa, transmitted via faecal contamination. Related coccidians from the genus *Eimeria* are common parasites of poultry, where they directly inhibit the uptake of essential dietary components, including carotenoids and other fat-soluble antioxidants in the gastrointestinal tract of chickens (e.g. Allen & Fetterer 2002), and consequently depress carotenoid-based pigmentation ('pale bird syndrome'; Tyczkowski, Schaeffer & Hamilton 1991). Ingested oocysts excyst in the epithelial cells of intestinal mucosa and liberate sporozoites from its contents. The sporozoites penetrate the cells of the host's small intestine and reproduce asexually. In the epithelial cells of the intestine, each generation of asexual reproduction produces multiple merozoites that infect new cells. This stage of the infection can result in the destruction of massive numbers of cells in the host's small intestine and, ultimately, lead to the host's death (e.g. Box 1977; Sironi 1994). Some of the merozoites that enter the host's cells transform into gametocytes. The gametocytes transform into gametes, the gametes fuse, and the resulting zygote begins to develop into an oocyst. The developing oocyst escapes from the host's cell, and it is passed in the host's faeces. In passerine birds, oocyst excretion has been observed already on the third day postinfection (Dolnik 2002). For the sake of brevity, we term the oocyst inoculum collected from a single host as a strain. It should be noted, however, that because the parasites undergo sexual reproduction in the host, the oocysts that pass out of hosts are not identical clones of the parasites already infesting the host. Thus the term 'strain' in this context denotes a mix of parasites, with the assumption that those from the same host are more closely genetically related than

those from different hosts (see also Williams 2002; Hõrak *et al*. 2006).

Importantly, coccidian infection intensity, measured as concentration of parasite oocysts in faeces, directly indicates parasite reproductive success (e.g. Chapman 1998). Thus, unlike in many other parasite models, proportional relationships between host resistance, parasite virulence and parasite fitness can be assumed. This assumption was validated in our study since individual changes in plasma triglyceride levels, body mass and infection intensities between different phases of the experiment were significantly correlated (Hõrak *et al*. 2006). The coccidian species present in the faeces of migrating Greenfinches in Estonia has been previously identified as *Isospora lacazei* (see Hõrak *et al*. 2004 for details). Since coccidian parasites are known to be highly host specific (e.g. Lillehoj & Trout 1993; Dolnik 2002), it was assumed that birds used in the current data set were infected with the same species of Isospora.

Because of diel periodicity in oocyst shedding (e.g. Brown, Ball & Holman 2001), faeces were collected in the evening. Two sheets of paper (paper bedding) were placed upon the sand bedding of cages 2 h before the lights were turned off. After the lights were turned off, the faeces were collected from the papers. Faecal samples were weighed to the nearest 0·01 g with an electronic balance (Mettler Toledo AB-S, Greifensee, Switzerland), suspended in 1 ml water and held at room temperature for 30 min. Then, the solution was drained through gauze into individual tubes and centrifuged at 1500 rpm (179 *g*) for 7 min. The supernatant was removed and 0·5 ml saturated NaCl water solution was added to the 0·5 ml residue. The number of oocysts was counted using the McMaster chamber (volume = 0·15 ml) and their concentration was expressed as the number of oocysts per gram of faecal sample. Repeatability of infection intensity, measured from two faecal samples collected at the same time, was 0·91 ($F = 20{\cdot}34$; $P < 0{\cdot}0001$; $N = 20$). During the pre-experimental period, coccidiosis was diagnosed from all the birds, with an average intensity of 105 918 ± 354 320 (SD) oocysts g^{-1}. Difference in individual infection intensities was very high, ranging from 266 ± 552 to 2 502 444 ± 1 415 898 oocysts g^{-1} (however, the second highest infection intensity was already considerably lower than the maximal, with an average pre-experimental oocyst count of 487 638 ± 804 029 oocysts g^{-1}).

Oocysts to be used for oral inoculations were collected during the 20-day period before the first blood sampling (days 6–26). Faecal samples of each bird were pooled to individual cell culture flasks with 75-cm^2 culture area and filter caps for continuous venting, and were preserved in 2% potassium dichromate ($K_2Cr_2O_7$) solution at room temperature and aerated daily. Sporulation of oocysts was registered 15 days after collection of the last sample (day 41), by microscopic observation. To prepare the inoculates, the mixture was drained through gauze and the resulting potassium dichromate solution containing oocysts centrifuged at 2500 rpm (496 *g*) for 10 min. After centrifugation, the supernatant was removed and 0·2 ml residue was resuspended in 1 ml water. This mixture was centrifuged again at 496 *g* for 10 min and the supernatant was removed leaving 0·2 ml residue. This washing procedure was repeated three or four times until the potassium dichromate was removed from the solution.

To describe the relationships between immune parameters and infection indices, Spearman rank correlations were used, since SRBC antibody titres are measured in the logarithmic scale. Sample sizes differ among some analyses because some PHA injections were unsuccessful and those birds were excluded from the analyses regarding PHA responses.

Results

Experimental infections with alien oocysts did not affect immune responses to novel antigens. Infection treatment groups did not differ significantly with respect to PHA response ($H = 3{\cdot}7$, $P = 0{\cdot}16$, $N = 28$) or SRBC antibody titres ($H = 2{\cdot}7$, $P = 0{\cdot}26$, $N = 36$; Kruskal–Wallis ANOVA for both tests; Table 1). Thus,

Table 1. Average trait values for the birds receiving different experimental infection treatments

	Experimental group*		
Variable: mean ± SD (*N*)	1	2	3
ln(pre-exp. oocyst count)	10·20 ± 1·73 (13)	7·39 ± 0·96 (13)	10·15 ± 1·53 (13)
ln(oocyst count at first exp. infection)	11·07 ± 2·03 (13)	9·22 ± 3·42 (13)	9·00 ± 3·07 (13)
ln(oocyst count at the end of experiment)	10·13 ± 1·70 (13)	7·74 ± 4·25 (12)	9·01 ± 3·34 (13)
SRBC antibody titre	2·08 ± 1·80 (13)	2·81 ± 1·53 (11)	1·91 ± 1·73 (12)
PHA response (mm)	0·19 ± 0·11 (10)	0·28 ± 0·12 (9)	0·16 ± 0·10 (9)
Body mass at the end of experiment (g)	30·58 ± 3·26 (13)	32·14 ± 3·31 (11)	31·64 ± 2·75 (12)
Plasma triglyceride concentration at the end of experiment (g/l)	2·15 ± 0·39 (13)	2·54 ± 0·54 (10)	2·29 ± 0·49 (11)

*Group 1: Infected two times with a mixture of oocysts from 6 donor individuals; Group 2: Infected first time with the same mixture as group 1, second time received water; Group 3: Infected first time with oocysts from the single donor individual; second time infected with own oocysts.

Table 2. Correlations between infection parameters measured at three stages (Fig. 1) of the study

Variable	Mean oocyst count at first exp. infection	Oocyst count at the end of experiment	Vulnerability to novel infections*
Mean pre-experimental oocyst count			
r_s	0·38	0·54	–0·19
P	0·015	0·0005	0·229
N	39	38	39
Mean oocyst count at first experimental infection			
r_s		0·78	0·74
P		<0·00001	<0·00001
N		38	39
Oocyst count at the end of experiment			
r_s			0·40
P			0·012
N			38

*Vulnerability to novel infections = difference in individual infection intensities measured during the peak phase of first experimental infection and during the whole pre-experimental period.

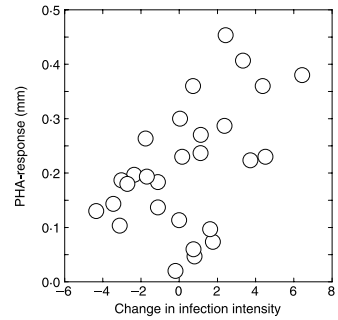

Fig. 2. Relationship between vulnerability to novel infection (change in infection intensity: (log)oocyst output after experimental infection – (log)pre-experimental oocyst output) and swelling response to PHA injection. $r = 0{\cdot}49$, $P = 0{\cdot}008$, $N = 28$.

in the following analyses, the treatment effects on the immune parameters were ignored.

During the 15 sampling days of the pre-experimental period, infection intensities of individual birds were moderately but significantly repeatable (repeatability = 0·40, $F_{38,545} = 11{\cdot}12$, $P < 0{\cdot}00001$). Average pre-experimental infection intensities correlated positively and significantly with infection intensities measured 2 months later during the peak antibody response (Table 2). Vulnerability to novel infections was quantified as a difference in individual infection intensities measured during the peak phase of first experimental infection and during the whole pre-experimental period. This parameter did not correlate significantly with pre-experimental infection intensity (Table 2). Expectedly, vulnerability to novel infections significantly predicted infection intensities at the end of the experiment, when SRBC antibody titres were measured (Table 2).

Infection intensities, measured at any of the three stages of the experiment (Fig. 1), did not correlate significantly with the PHA response ($r_s = –0{\cdot}30$ ... $0{\cdot}33$, $P = 0{\cdot}082$ … $0{\cdot}71$, $N = 28$). However, birds that responded to new infection with an increase in infection intensities mounted a stronger swelling response to PHA injection (Fig. 2). SRBC antibody titres did not correlate with vulnerability to novel infections ($r_s = 0{\cdot}21$, $P = 0{\cdot}23$, $N = 36$) or PHA response ($r_s = 0{\cdot}13$, $P = 0{\cdot}52$, $N = 28$).

Relationships between SRBC titres, infection intensities and condition indices were different for individuals who responded to the first infection with increased oocyst production and for those who did not (Fig. 3). Among birds that were not vulnerable to novel infections, infection intensities correlated negatively and condition indices correlated positively with antibody titre. However, no clear patterns emerged among the birds whose infection intensity increased after the first experimental infection (Fig. 3). When data were pooled over all individuals, only plasma triglyceride levels correlated significantly with antibody titre ($r_s = 0{\cdot}49$, $P = 0{\cdot}003$, $N = 34$). None of the condition indices correlated significantly with PHA response ($r_s = 0{\cdot}21$ … $0{\cdot}31$, $P = 0{\cdot}11$ … $0{\cdot}28$, $N = 26$ … 28).

Discussion

In this study we used two different measures to characterize between-individual differences in their infection status. Infection intensities measured before the experimental inoculations were significantly repeatable during the 1-month period and also correlated positively with individual infection intensities measured 2 months later. Thus, consistent between-individual differences in parasite loads clearly persisted despite the various manipulations performed during our study, and new inoculations with parasites of different origin. Thus, at least to a certain extent, infection intensities measured at any moment in the study can be considered to reflect differential abilities of individual birds to control their parasite loads. Additionally, we could distinguish another aspect of disease resistance on the basis of whether intensity of parasitaemia increased or decreased after reinfection with novel strains, compared with the average pre-experimental infection level. This response, termed as vulnerability to new infections, evidently characterizes a different aspect of individual parasite resistance because its values did not correlate significantly with average pre-experimental infection levels.

Magnitude of swelling response to PHA injection correlated significantly with only one of our measures of parasite resistance. Birds that were more vulnerable to the experimental infection with new parasite strains mounted stronger immune responses (Fig. 2). Thus, if we assume that the magnitude of swelling response to

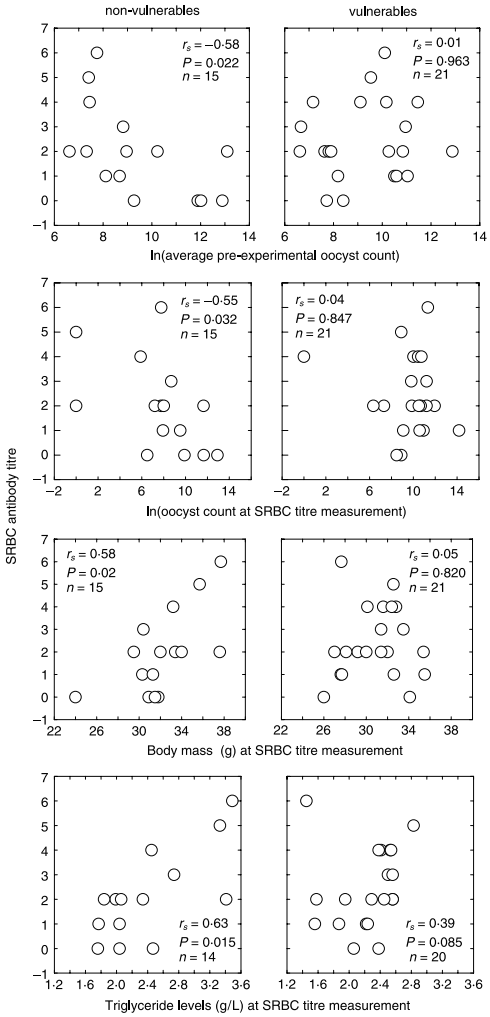

Fig. 3. Relationships between SRBC antibody titre *vs* pre-experimental infection intensity, infection intensity at titre measurement, body mass and plasma triglyceride concentration. Left panel: non-vulnerable individuals whose infection intensities did not increase after experimental infection with novel strains. Right panel: vulnerable individuals.

PHA indeed reflects the animal's general potential for cell-mediated immunity (e.g. Smits *et al*. 1999; Martin *et al*. 2001), then it would appear that the most immunocompetent individuals are least resistant to a real infections! However, such an interpretation would be overly simplistic, given the integrated and extremely complex structure of the vertebrate immune system. As pointed out by Adamo (2004), correlations between assays of immunity and disease resistance can be pathogen dependent. In the context of the current study, it is important to note that immune response against coccidiosis is mainly based on activation of various cell populations, including T lymphocytes, NK cells and macrophages (Lillehoj & Trout 1996). During this process, cytokines, including IFN-γ, TNF-β and IL-2, are produced, which are also responsible for the delayed-type hypersensitivity reactions induced by the PHA injection (e.g. Lillehoj 1998). It is therefore likely that birds that were vulnerable to the experimental reinfection were actively fighting against it and had therefore up-regulated their T-cell mediated immune function. Similar enhancing effect of parasites on T-cell mediated immune function has previously been shown for House Martins (*Delichion urbica*) experimentally infected with the haematophagus bug *Oeciacus hirundinis* (Christe *et al*. 2000). Analogously, Gwinner, Oltrogge & Nienaber (2000) found higher PHA responses in nestling Starlings (*Sturnus vulgaris*) from nests with high ectoparasite loads, while experimental infection of chickens with the bacterium *Salmonella typhimurium* resulted in higher lymphocyte proliferative responses to mitogens and increased cytotoxic activity of NK cells (Lessard, Hutchings & Spencer 1995). These examples call for caution when interpreting the results of standard immune tests in the context of parasite resistance (see also Tella *et al*. 2002; Ryder 2003; Adamo 2004). This caution is perhaps especially required in the case of PHA responses, which do not represent a single clearly definable immune phenomenon but a suite of responses that result in swelling (Matson, Ricklefs & Klasing 2005).

The relationship between SRBC antibody titres, infection intensities and condition indices was different for individuals whose intensity of parasitaemia increased after reinfection with novel strains (vulnerables) and for those whose oocyst output declined (non-vulnerables; Fig. 3). Among non-vulnerables, infection intensities correlated negatively with SRBC antibody titre, while no such pattern emerged among the birds whose infection intensity increased after first infection. At the same time, among non-vulnerables, SRBC titres also clearly reflected their nutritional state (Fig. 3), correlating positively with body mass and plasma triglyceride levels. Coccidian infections are known to cause decline in individual nutritional status (Hõrak *et al*. 2004) by damaging intestinal absorptive mucosa (e.g. Ruff & Fuller 1975; Hoste 2001). It thus seems that those non-vulnerable birds who had less damaged intestine (were less inflicted by or more resistant to coccidian infection) were in better nutritional condition and thus capable to raise a stronger humoral immune response against a novel antigen. From here we might speculate that birds who had the lowest pre-experimental oocyst counts and did not respond to experimental infection were in fact resistant to those Isosporan strains obtained by experimental inoculation.

Among such birds, then, a capability to respond to immune challenge with a novel multigenic antigen such as SRBC thus truly reflected some component of parasite resistance and general condition. Lack of such correlations among birds that were vulnerable to novel coccidian infection, however, remains puzzling. It should be noted in this context that we were unable to assess the immune function of birds before they became infected, and therefore we could only register the effect of ongoing parasitic infection on the expression of immune parameters. We cannot thus exclude the possibility that postnatal selection for parasite resistance and/or immunoresponsiveness had eliminated the most susceptible individuals from the sample available for our experiment. This, in turn, might have obscured the expected positive relationships between parasite resistance and immunocompetence in our study. Furthermore, our SRBC assay did not enable us to separate the proportional contributions of IgM and IgG to overall haemagglutination activity. It therefore remains possible that correlations between specific immunoglobulin levels and indices of parasitism and physiological condition of birds would have differed from correlations with overall serum haemagglutination activity assessed in our experiment.

In conclusion, at least for a subsample of studied Greenfinches, high anti-SRBC antibody titres (unlike the PHA responses) indeed reflected the superior resistance to an important parasite species. The causality of this relationship cannot, however, be currently inferred from our data. One possibility is that better general immunocompetence (indicated by high SRBC titres) enabled some birds to suppress the challenge infections more efficiently, which also resulted in higher body mass and plasma triglyceride levels, owing to their less damaged intestinal mucosa. Alternatively, genetic resistance would enable some individuals to manage their challenge infections so efficiently that this results in better nutritional condition. Better condition, in turn, would enable them to mount stronger antibody response against SRBC. Another important finding of our study is that correlations between a standard measure of immunocompetence and various condition indices became detectable only after the birds were divided into 'vulnerables' and 'non-vulnerables' on the basis of their ability to resist novel parasite strains. This stresses the importance of measuring host infection resistance in immunoecological studies. Altogether, these results exemplify the diversity of outcomes when a host's immune system is simultaneously challenged by natural enemies and artificial antigens.

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IV

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Antioxidant protection, carotenoids and the costs of immune challenge in greenfinches

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Summary

Costs accompanying immune challenges are believed to play an important role in life-history trade-offs and warranting the honesty of signal traits. We performed an experiment in captive greenfinches (*Carduelis chloris* L.) in order to test whether and how humoral immune challenge with non-pathogenic antigen [sheep red blood cells (SRBC)] affects parameters of individual condition including intensity of coccidian infection, estimates of total antioxidant protection, plasma carotenoids and ability to mount a cell-mediated immune response. We also asked whether the potential costs of immune challenge can be alleviated by dietary carotenoid supplementation. None of the treatments affected intensity of coccidiosis. Humoral immune challenge suppressed the cell-mediated response to phytohemagglutinin (PHA), suggesting a trade-off between the uses of different arms of the immune system. Immune challenge reduced body-mass gain, but only among the carotenoid-depleted birds, indicating that certain somatic costs associated with immune system activation can be alleviated by carotenoids. No evidence for oxidative stress-induced immunopathological damages could be found because immune activation did not affect total antioxidant protection or carotenoid levels. Carotenoid supplementation inclined birds to fattening, indicating that lutein interfered with lipid metabolism. Altogether, our results support the hypotheses of biological importance of carotenoids and exemplify the overwhelming complexity of their integrated ecophysiological functions.

Key words: immune challenge, phytohemagglutinin, plasma carotenoids, SRBC, total antioxidant capacity.

Introduction

Actual and potential damage caused by parasites and pathogens has led host species to develop sophisticated protection mechanisms, ranging from behavioural defences and physical barriers to the immune system. Using the immune system can be costly, as revealed by studies demonstrating the adverse effects of activation of some components of immune defence on reproductive effort (e.g. Ilmonen et al., 2000), ornamental trait expression (e.g. Kilpimaa et al., 2004), cognitive performance (e.g. Mallon et al., 2003), other components of immune defence (Kidd, 2003) and even survival (Moret and Schmid-Hempel, 2000; Victor and de la Fuente, 2003; Hanssen et al., 2004). However, the question as to what exactly makes the activation of immune defences costly has remained less clearly understood (e.g. Schmid-Hempel, 2002; Zuk and Stoehr, 2002). The traditional view of ecologists, namely that the costs involved in life-history trade-offs are basically energetic, has gained equivocal support in immunoecological studies (reviewed by Råberg et al., 2002; Demas, 2004; Eraud et al., 2005); an alternative hypothesis is that costs of immune responses are primarily caused by the accompanying immunopathological damages (Råberg et al., 1998; Westneat and Birkhead, 1998).

An important source of immunopathology is oxidative stress, caused by excess production of reactive compounds during immune responses (Halliwell and Gutteridge, 1999). Oxidative products and free radicals, which are highly reactive by-products of normal metabolism and immune defences, can cause extensive damage to nucleic acids, proteins and lipids if an organism lacks sufficient antioxidant protection (Halliwell and Gutteridge, 1999). To control and neutralise free radicals, animals maintain a system of defences based on different antioxidants. Endogenous antioxidants (like uric acid, bilirubin and albumin, and enzymes such as catalase, superoxide dismutase and glutathione peroxidase) are synthesized by an organism whereas exogenous antioxidants (like vitamins E and A, and carotenoids) must be obtained from food.

Of all the antioxidants, animal ecologists have paid a disproportionate amount of attention to carotenoids (Lozano, 1994; Olson and Owens, 1998; von Schantz et al., 1999; Møller et al., 2000; McGraw, 2006). Carotenoid-based visual characters enable individuals to signal their phenotypic and/or

genetic quality: if an individual has only a limited amount of carotenoids at its disposal, then it can use them for signals only when it does not need them for maintenance (Lozano, 1994). Hence, carotenoid-based traits might either signal foraging (and food absorption) efficiency, immunocompetence or anti-oxidative potential of signallers. The relative importance of these factors is currently under lively debate (Hill, 1999; Lozano, 2001; Hartley and Kennedy, 2004).

Compared with their role in signalling and immunity, the antioxidant function of carotenoids has remained much more poorly understood, even in traditional mammal models (El-Agamey et al., 2004). The situation is even more complicated with birds. Given that most avian species live longer than similar-sized mammals despite their higher metabolic rates, birds are thought to have evolved unique protective mechanisms against oxidative damage (Klandorf et al., 2001). With few exceptions (e.g. Woodall et al., 1996; Jaensch et al., 2001), antioxidant properties of carotenoids in birds have been predominantly studied in the context of embryo-protective maternal effects (Surai, 2002; McGraw et al., 2005a), and only few studies (Alonso-Alvarez et al., 2004; Bertrand et al., 2006; Costantini et al., 2006) have measured the relationships between carotenoids and general antioxidant defences in nestlings or adults.

Here we address the questions about the role of carotenoids in modulation of oxidative stress *via* changes of total antioxidative potential and physiological consequences of immune challenges in captive greenfinches. (1) Under the hypothesis that activation of the immune system by a novel antigen weakens antioxidant protection, we predicted that an immune challenge results in a reduction of plasma carotenoid levels and total antioxidant protection. If mounting an immune response impairs the general physiological condition of an individual, we also expected to find an effect of immune challenge on the indices of individual nutritional state. (2) If a trade-off exists between different arms of the immune system, so that eliciting a humoral response diverts resources away from the cell-mediated response, we predicted that birds injected with sheep red blood cells (SRBC) will produce a weaker cutaneous swelling in response to mitogen [phytohemagglutinin (PHA)] injection. This prediction is based on the allocation principle, which underlies the rationale of ecological immunology (e.g. Sheldon and Verhulst, 1996), and on the evidence regarding cross-regulation between the different components of the immune system from mammal models (e.g. Kidd, 2003). (3) Under the hypothesis that carotenoids are involved in antioxidant protection and general health maintenance, we predicted that these potential costs of immune challenge (i.e. reduced antioxidant protection and nutritional state) will be alleviated among the birds receiving dietary carotenoid supplementation. (4) We also predicted that carotenoid-supplemented birds mount a stronger immune response against foreign antigens than control individuals if carotenoids exert an immunostimulatory effect in our model system. (5) Finally, assuming that carotenoids significantly contribute to total antioxidativity, we predicted that individual plasma-carotenoid levels correlate positively with measures of total antioxidant capacity.

Materials and methods

Greenfinches weigh approximately 30 g and are sexually dichromatic, gregarious granivorous passerines native to the western Palearctic region. Carotenoid-based plumage coloration in males is sexually selected (Eley, 1991) and negatively affected by intestinal (Hõrak et al., 2004), viral (Lindström and Lundström, 2000) and hematozoan (Merilä et al., 1999) infections. Fifty-six male greenfinches were caught in mist-nets at the Sõrve Bird Observatory (Saaremaa, Estonia; 57°55′N; 22°03′E) on 25 (day 0) and 26 January 2005. At capture, 13 of these individuals were blood sampled during the first morning hours to obtain natural background levels for the concentration of carotenoids. Birds were transported to Tartu and housed in individual indoor cages (27×51×55 cm) with sand bedding. Mean temperature in the aviary during the experiment was 14.6±1.2°C (s.d.) and mean humidity was 55.5±7.6% (s.d.). The birds were supplied *ad libitum* with sunflower seeds and filtered tap water. Birds were held on the natural day-length cycle. All blood samples were collected before the lights were turned on, in order to obtain the values of biochemical parameters characteristic to the state of overnight fast. Birds were released to their natural habitat on 19 March 2005 (day 53). The study was conducted under license from the Estonian Ministry of the Environment.

Experimental procedure

The experimental procedure is described in Fig. 1. After transportation to Tartu, birds were allowed a 13-day acclimatisation period (days 2–15). Birds were divided into four equal (14 birds) treatment groups that were set to have similar average body mass at capture and age composition (five first-year and nine older birds in each group). On the morning of day 15, pre-experimental blood samples were collected and the birds were assigned to 2×2 treatments by immune challenge and carotenoid supplementation. The immunochallenged group (28 birds) received an injection of 50 μl of 40% suspension of SRBC in isotonic saline into the pectoralis muscle on days 15 and 31 (Fig. 1). Controls were injected with the same amount of isotonic saline only. Half the birds started to receive carotenoid supplementation in their diet on the same day. Supplementation consisted of a 10 $\mu g\ ml^{-1}$ water solution of lutein and zeaxanthin (20:1, w/w), prepared from OroGlo liquid solution of 11 $g\ kg^{-1}$ xanthophyll activity (Kemin AgriFoods Europe, Herentals, Belgium). These solutions were freshly prepared each morning using filtered (Brita® Classic; BRITA GmbH, Taunusstein, Germany) tap water at 4°C and were provided in 30-ml doses in opaque dispensers in order to avoid oxidation of carotenoids. Control birds received filtered tap water.

On day 21 (seven days after treatments started), a second blood sample was collected (‘First titre’ in Fig. 2). On the next day, all the birds were injected intradermally in the wing web

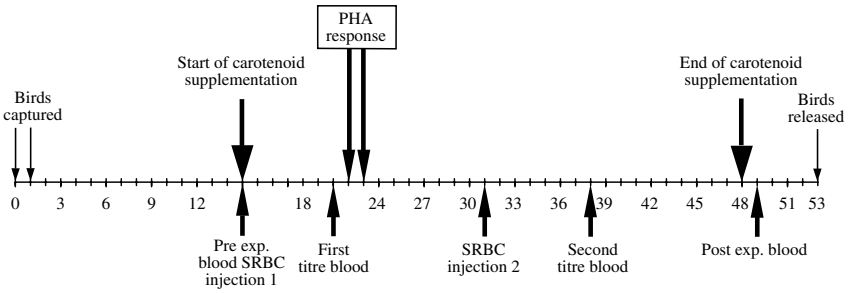

Fig. 1. Experimental process. Day 0=25 January 2005.

with 0.2 mg of PHA in 0.04 ml of isotonic saline. The swelling response was measured 24 h later. On day 31 (10 days after primary immunisation), previously immunised birds were again injected with the same dose of SRBC and control birds received a second saline injection. Blood for secondary-antibody titres ('Second titre' in Fig. 2) was collected eight days after secondary immunisation (Fig. 1). Carotenoid supplementation was ended on the 48th day of the experiment and the last ('Post exp.') blood sample was collected the following day (Fig. 1). In all cases, 100–300 μl of blood was drawn from brachial or tarsal veins and, after centrifugation, serum (for antibody measurement) and plasma (for the remaining analyses) were stored at –75°C until analysed. All spectrophotometric analyses were performed with a Tecan microplate reader (Model Sunrise; Tecan Austria GmbH, Grödig/Salzburg, Austria). During the few hours between

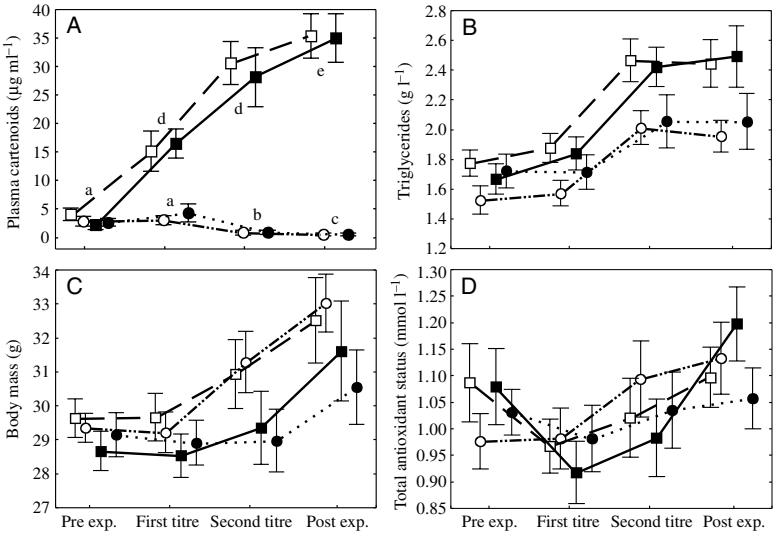

Fig. 2. Effects of carotenoid supplementation and immune challenge on physiological parameters of greenfinches. For carotenoids, individual changes are tested with Wilcoxon matched-pairs tests and between-treatment differences with Mann–Whitney *U*-tests. Carotenoid concentrations, marked with different letters, are significantly different from each other ($P<0.05$). Group sizes range from five to eight on the first-titre measurement and from 11 to 14 on other occasions. Mean-trait values, exact sample sizes and statistics are presented in Table 1. For other variables, *P*-values for time × treatment interaction terms are presented in the text, and in most cases each group contains 14 individuals. Open squares, carotenoid–saline; filled squares, carotenoid–SRBC; filled circles, water–SRBC; open circles, water–saline.

blood collection and centrifugation, samples were maintained in a refrigerator at 4°C.

In the course of study, birds were monitored for their individual levels of coccidian infection by fecal examination. The coccidian species present in the feces of migrating greenfinches in Estonia has been previously identified as *Isospora lacazei* (for details, see Hõrak et al., 2004). Coccidian-infection intensities (number of oocysts per gram of feces) of individual greenfinches were quantified as described previously (Hõrak et al., 2004; Hõrak et al., 2006; Saks et al., 2006). Infection intensities were determined on days 4, 6, 8, 10, 12, 16, 18, 20–22, 24–34, 36, 37, 39, 41, 44, 47 and 48 of the experiment. All the birds carried infections when brought into the aviary. On days 24–30, all the birds received sulphonamide-coccidiostatic treatment (sulfathiazole sodium pentahydrate, 2 g l^{-1} water) in their drinking water in order to equalise their infection status. The treatment, however, was not fully efficient because by the end of treatment (day 25), 27% (15/56) of the birds were still shedding oocysts. The effect of treatment almost completely vanished by day 34, when 95% of birds (53/56) had started to shed oocysts again.

Assessment of immune function

Immune response against SRBC involves both B- and T-lymphocytes and is used for routine evaluation of humoral immunity in immunological, immunotoxicological and ecological studies (see Hõrak et al., 2003). Anti-SRBC antibody titres were measured using a hemagglutination assay (Wegmann and Smithies, 1966; Lawler and Redig, 1984) as described in detail by Saks et al. (Saks et al., 2003), with the exception that 25 μl of serum and 25 μl of isotonic saline were pipetted into the first well of a microtitre plate. This mixture was serially diluted using 25 μl of saline. Hence, we used initial plasma concentrations that were four times higher than in our previous studies (Hõrak et al., 2003; Saks et al., 2003), which enabled us to detect antibody concentrations that were four times lower than previously. (Yet no antibodies were detected in the serum of non-immunized birds.) Titre was scored as the number of wells in a dilution row that contained a sufficient amount of antibodies to hemagglutinate SRBC.

Cutaneous hypersensitivity reaction, resulting from PHA injection, reflects the combined responses of T-cells, cytokines and inflammatory cells (e.g. Stadecker et al., 1977). This assay has become increasingly popular in avian studies, where it is considered as a proxy of cell-mediated immune responsiveness (see Smits et al., 1999). We followed the simplified protocol (Smits et al., 1999) as described in detail by Saks et al. (Saks et al., 2003). The repeatability (Lessells and Boag, 1987) of swelling response, based on three consecutive measurements, was 0.88 (F=22.9; P<0.0001; N=49).

Indices of nutritional state

To assess nutritional state, we measured body mass (before blood samplings) and plasma triglyceride concentrations. High blood triglyceride levels are indicative of a resorptive state during which lipid is formed by the liver and deposited in muscle and adipose tissues. Hence, triglyceride concentrations reflect the individual's state of fattening by indicating the amount of lipids absorbed during the few hours before blood sampling (Jenni-Eiermann and Jenni, 1998). Blood was collected after nocturnal fast, so plasma triglyceride levels in our study reflect the variation in basic nutritional state, independently of recent food intake. Concentrations were determined by enzymatic colorimetric test (GPO-PAP method) (Human GmbH, Wiesbaden, Germany).

Carotenoids

The most prevalent carotenoids in the plasma of greenfinches are lutein and its structural isomer, zeaxanthin (McGraw, 2004). Concentrations of carotenoids were determined spectrophotometrically (e.g. Tella et al., 1998; Bortolotti et al., 2000; Peters et al., 2004) using acetone-resistant microtitre plates. Acetone (150 μl) was added to 15 μl of plasma and centrifuged for 10 min at 16 800 ***g***. Absorbance of supernatant was measured at 449 nm, corresponding to the maximum absorbance of lutein in acetone (Zsila et al., 2005). Calibration curves were prepared using lutein (X-6250; Sigma, St Louis, MO, USA) as standard. Repeatability (Lessells and Boag, 1987) of carotenoid measurements between different microtitre plates was 0.95 ($F_{15,20}$=46.0; P<0.0001).

Total antioxidativity

Two methods, based on the capacity of biological fluids to inhibit redox reaction induced by free radicals, were used for assessment of total antioxidant capacity of plasma. A total antioxidant status (TAS) assay was performed, adapting the commercially available kit (Randox Laboratories, Crumlin, UK) for small (5 μl) plasma samples. This assay (sometimes also termed TEAC) is widely used in clinical studies (Dotan et al., 2004). In this assay, azino-diethyl-benzthiazoline sulphate (ABTS) is incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^+$. This has a relatively stable blue-green colour, which is measured at 600 nm. Antioxidants in the plasma cause suppression of this colour production to a degree that is proportional to their concentration. TAS is expressed in mmol l^{-1}. Repeatability (Lessells and Boag, 1987) of TAS values among individual samples, measured on different plates, was 0.93 ($F_{14,15}$=29.2; P<0.0001).

Total antioxidant potential (AOP) was estimated using the BIOXYTECH® AOP-490™ assay (OxisResearch™, Portland, OR, USA), which is based upon the reduction of Cu^{2+} to Cu^+ by the combined action of all antioxidants presented in a sample. A chromogenic reagent, bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline), selectively forms a 2:1 complex with Cu^+, which has a maximum absorbance at 490 nm. Colour change of the plasma, incubated with reagent containing Cu^{2+} and chromogen for 3 min at room temperature, is measured. A standard of known uric acid (a water-soluble antioxidant) concentration was used to create the calibration curve, so the results are quantified in mmol l^{-1} uric acid equivalents. The assay was adapted for small (5 μl) plasma samples.

Repeatability of AOP values among individual samples, measured on different plates, was 0.88 ($F_{14,14}$=15.2; P<0.0001).

Statistics

Effects of experimental treatments on the dynamics of body mass, triglycerides and TAS were analysed by repeated-measures analysis of variance (ANOVA), dropping non-significant interaction terms from the final models. Assumptions for the parametric analyses were met for these variables. Since carotenoid concentrations were not normally distributed among unsupplemented birds, we could not apply repeated-measures ANOVA for testing the treatment effects. Therefore, individual changes within treatment groups were tested with Wilcoxon matched-pairs tests and between-treatment differences with Mann–Whitney *U*-tests (Table 1). Age (first-year *versus* older) did not affect any of the studied parameters. Non-parametric tests were applied for analyses of coccidian-infection intensities because these were not normally distributed. *P*-values are for two-tailed tests. Sample sizes differ between some analyses because of our inability to collect a sufficient amount of blood from all the birds. Mean trait values are presented with ± s.d.

Results

Physiological effects of carotenoid supplementation and immune challenge

Plasma carotenoid concentration at capture ranged from 1.5–21.6 μg ml^{-1}, averaging at 10.2±6.6 μg ml^{-1} (N=13). After 15 days spent in captivity, average carotenoid levels in greenfinches had decreased more than twofold (to 3.8±2.7 μg ml^{-1}) as compared with the levels at capture (Z=3.1, P=0.002, Wilcoxon matched-pairs test). During the experiment, plasma carotenoid levels in unsupplemented birds continued to decline, whereas among the supplemented birds, average plasma-carotenoid concentrations steadily increased (Fig. 2A). Immunisation with SRBC had no effect on the dynamics of plasma carotenoid levels in either group (P=0.5–1; see Table 1 for details).

Carotenoid supplementation increased plasma-triglyceride levels in the second half of the experiment; however, triglycerides were not affected by the immune challenge [Fig. 2B; $F_{3,141}$=3.33, P=0.021 for time × carotenoid interaction term and $F_{3,141}$=0.11, P=0.952 for time × SRBC interaction term in repeated-measures ANOVA with main effects of carotenoid ($F_{1,47}$=8.46, P=0.005) and SRBC ($F_{1,47}$=0.194, P=0.662) treatments and time ($F_{3,141}$=44.17, P<0.0001)].

Body mass of birds increased during the second half of the experiment (Fig. 2C). Mass dynamics in the whole sample was not affected by carotenoid supplementation or immunisation treatments [$F_{3,141}$=1.53, P=0.210 for time × carotenoid interaction term and $F_{3,141}$=0.35, P=0.786 for time × SRBC interaction term in repeated-measures ANOVA with main effects of carotenoid ($F_{1,47}$=0.03, P=0.865) and SRBC ($F_{1,47}$=0.05, P=0.824) treatments and time ($F_{3,141}$=5.66, P=0.001)]. However, the effect of immunisation on body-mass dynamics emerged when unsupplemented birds were analysed separately ($F_{3,78}$=10.7, P=0.008 for time × SRBC interaction term in repeated-measures ANOVA with main effects of SRBC treatment ($F_{1,26}$=1.9, P=0.193) and time ($F_{3,78}$=43.3, P<0.00001)]. This was because immunised birds gained body mass more slowly than non-immunised birds (Fig. 2C).

None of our treatments affected plasma TAS [Fig. 2D; $F_{3,141}$=1.52, P=0.209 for time × carotenoid interaction term and $F_{3,141}$=0.35, P=0.785 for time × SRBC interaction term in repeated-measures ANOVA with main effects of carotenoid and SRBC treatments ($F_{1,46}$=0.03–0.05, P=0.8) and time ($F_{3,141}$=5.67, P=0.001)]. Similarly, plasma AOP did not differ between treatments after secondary immunisation ($F_{3,52}$=0.47, P=0.701) or at the end of experiment ($F_{3,37}$=0.47, P=0.705).

Swelling response to PHA injection tended to be lower among SRBC-injected birds (0.34±0.18 mm, N=26) than among unsupplemented birds (0.45±0.23 mm, N=23; t=1.90, P=0.064). This effect of treatment became significant ($F_{1,45}$=4.76, P=0.034) after inclusion of body mass at capture [$F_{1,45}$=7.77, P=0.008, β=0.37±0.13 (s.e.m.)] as a covariate into an analysis of covariance (ANCOVA) model.

Table 1. *Mean plasma carotenoid concentrations (μg ml^{-1}) in different treatment groups and P-values and Z-statistics for between-group comparisons (Mann–Whitney U-tests)*

	Control			Carotenoid			Difference between control and carotenoid
	SRBC	Saline		SRBC	Saline		
Date	Mean ± s.d. (N)	Mean ± s.d. (N)	P diff. (Z)	Mean ± s.d. (N)	Mean ± s.d. (N)	P diff. (Z)	P diff. (Z)
9 Feb	2.6±2.3 (13)	2.8±3.2 (14)	0.846 (0.19)	2.2±2.9 (14)	4.1±4.2 (14)	0.206 (1.26)	0.859 (0.17)
15 Feb	4.3±3.8 (6)	3.1±.3 (8)	0.796 (0.25)	16.5±6.3 (6)	15.1±7.9 (5)	0.715 (0.36)	0.00005 (4.05)
4 March	0.9±1.1 (12)	0.8±1.5 (12)	0.644 (0.46)	28.2±16.3 (10)	30.6±13.6 (13)	0.660 (0.43)	<0.00001 (5.51)
15 March	0.5±0.7 (12)	0.5±0.9 (14)	0.554 (0.59)	35.0±14.7 (13)	35.4±12.8 (11)	0.950 (0.06)	<0.00001 (5.98)

Individual changes in carotenoid concentrations among control birds: 9 Feb–15 Feb: P=0.362; Z=0.91; N=14. 15 Feb–4 March: P=0.006; Z=2.75; N=11. 4 March–15 March: P=0.004; Z=2.84; N=23. Individual changes in carotenoid concentrations among carotenoid-supplemented birds: 9 Feb–15 Feb: P=0.003; Z=2.93; N=11. 15 Feb–4 March: P=0.138; Z=1.48; N=9. 4 March–15 March: P=0.006; Z=2.73; N=19. Wilcoxon matched-pairs tests.

Pre-experimental coccidian-infection intensity (averaged over first five days when infection was measured) did not differ between the experimental groups (H=0.37, N=56, P=0.946; Kruskal–Wallis ANOVA). Infection intensities at the end of the experiment (averaged over days 47–48) were not affected by the carotenoid treatment (Z=0.4, N=55; P=0.711; Mann–Whitney U-test) or immune challenge (Z=0.4, N=55; P=0.686; Mann–Whitney U-test).

Effects of carotenoid supplementation on the immune responses

Twenty-four out of 28 immunised birds produced a detectable amount of antibodies against SRBC after primary immunisation, and 26 out of 28 birds responded to secondary immunisation. We could not detect a difference in the magnitude of immune responses between carotenoid-supplemented and unsupplemented birds (Z=1.65 and 0.96, P=0.10 and 0.33, for primary and secondary titres, respectively). Furthermore, after the exclusion of nonresponsive individuals (i.e. the birds with 0-titres) from the sample, primary immune response was significantly higher in the unsupplemented group (6.76±1.21, N=12 *versus* 5.50±1.39, N=12; t=2.35, P=0.028).

Swelling response to PHA injection did not differ significantly between supplemented (0.42±0.23 mm, N=26) and unsupplemented birds (0.38±0.19 mm, N=23; t=0.77, P=0.445). Inclusion of the SRBC immunisation as a factor in the ANOVA model did not affect the significance of the carotenoid-treatment term. PHA response did not correlate with individual plasma-carotenoid levels (r_s=0.06–0.11; P=0.6).

Correlations between indices of total antioxidativity, carotenoids and infection

Our two measures of antioxidant protection, TAS and AOP, correlated positively in all three measuring occasions (r=0.52–0.81; P=0.015–<0.0001; N=12–56; Fig. 3). However, none of these measures correlated significantly with plasma carotenoid levels (r=–0.10–0.19; P=0.2–1.; N=25–55). High pre-experimental infection intensities were accompanied by low plasma-carotenoid levels (r_s=–0.36; N=55; P=0.007). None of the other health parameters correlated significantly with pre-experimental (r_s=–0.05–0.08; P=0.5–0.9; N=56) or post-experimental (r_s=–0.07–0.09; P=0.6–0.9; N=41–55) infection intensities.

Discussion

Dietary manipulation of carotenoid availability in our experiment was effective, resulting in almost total depletion of plasma carotenoid levels in the unsupplemented group. After 33 days of dietary supplementation, mean carotenoid concentrations of supplemented birds exceeded those of unsupplemented birds by approximately 70-fold. By the end of the experiment, plasma carotenoid levels among the supplemented birds were more than three times higher (35 μg ml^{-1}) than carotenoid levels at capture (10 μg ml^{-1}).

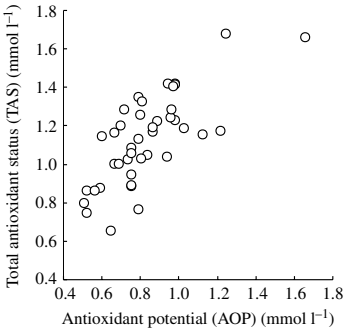

Fig. 3. Relationship between two measures of plasma antioxidant activity (TAS and AOP) at the end of the experiment (r=0.75; P<0.00001; N=41).

However, we are confident that such concentrations remain within the physiological range because the chroma of tail feathers, plucked and regrown during the experiment, remained approximately 10% lower than chroma of naturally grown feathers (U.K., L.S. and P.H., unpublished data). This indicates that plasma carotenoid levels of free-living greenfinches during moult exceed those obtained in this study. Relatively low carotenoid levels at capture in our study can probably be explained by the diet at capture site, where the birds predominantly fed on supplemented sunflower seeds, which have extremely low carotenoid content (McGraw et al., 2001).

Costs of immune response

Assuming that use of the immune system is costly, we predicted that mounting an immune response against SRBC would result in reduced antioxidant protection and plasma carotenoid levels, as well as impaired physiological condition and cell-mediated immunoresponsiveness. Of these parameters, the immune activation most clearly affected the swelling response to PHA. Adjusting for between-individual variation in physiological condition (estimated on the basis of body mass at capture), birds immunized with SRBC produced weaker cutaneous swelling response to PHA than sham-immunised birds on the seventh day after SRBC injection. This result points to the possible trade-off between the use of the different arms of the immune system, which is compatible with the general logic of ecological immunology (e.g. Sheldon and Verhulst, 1996; Zuk and Stoehr, 2002). This trade-off might be rooted in the basis of cross-regulation between humoral and cell-mediated immune responses (Mosmann and Coffman, 1989) in which (humoral) Th2 responses exert anti-inflammatory action by negatively regulating Th1-cell-mediated immunity (and *vice versa*). Although such cross-regulations have been frequently observed in mammal models (reviewed by Kidd, 2003), the discovery of a suppressed cutaneous swelling response in response to humoral immune

system activation in greenfinches is, to our knowledge, the first such evidence in birds. The generality of this phenomenon, however, is unclear as no suppression of PHA response by SRBC injection was observed in nestling western bluebirds (*Sialis mexicana*) (Fair and Myers, 2002) or growing quail (*Coturnix coturnix japonica*) (Fair et al., 1999).

Another piece of evidence about the possible costs of humoral immune activation originates from the data about body-mass dynamics. SRBC challenges significantly slowed down the increase of body mass during the second half of the experiment, but only for birds in the carotenoid-free diet (Fig. 2C). The findings of reduced body mass, mass gain or growth in response to non-pathological immune challenge have been documented in several avian studies (e.g. Klasing et al., 1987; Fair et al., 1999; Ots et al., 2001; Bonneaud et al., 2003) (but see Whitaker and Fair, 2002; Hõrak et al., 2000; Hõrak et al., 2003). Possible mechanisms include energy reallocation from maintenance to immune function (reviewed by Lochmiller and Deerenberg, 2000; Demas and Sakaria, 2005) or inflammation-induced sickness syndrome, which results in reduced food intake and locomotory activity (e.g. Bonneaud et al., 2003; Klasing, 2004). In this context, our result regarding the lack of effect of immune challenge on body-mass dynamics among carotenoid-supplemented birds is particularly interesting because it suggests that some physiological costs associated with immune system activation can be alleviated by the carotenoid supplementation. This effect was probably related to the enhancement of fat deposition among carotenoid-fed birds (Fig. 2B).

Assuming that possible immunopathological effects of SRBC challenge emerge because of excessive reactive-species production, we expected immunisation treatment to affect the biomarkers of antioxidant protection. However, despite the above-mentioned physiological effects of immune challenge, we did not detect any carotenoid depletion among SRBC-injected birds (Fig. 2A). This result is inconsistent with Alonso-Alvarez et al. (Alonso-Alvarez et al., 2004), who showed that immune challenge with a bacterial lipopolysaccharide (LPS), significantly depressed plasma carotenoid levels in captive zebra finches (*Taeniopygia guttata*). Similar results were obtained with chickens (*Gallus gallus domesticus*), where carotenoid depletion from the plasma and other tissues was specifically associated with markers of acute-phase response, such as interleukin-1 (Koutsos et al., 2003). In mallards (*Anas platyrhynchos*), higher anti-SRBC antibody titres were associated with a greater decline of plasma carotenoids (Peters et al., 2004). More generally, reduced levels of plasma lutein have also been associated with markers of inflammation in human studies (e.g. Kritchevsky et al., 2000; Gruber et al., 2004).

Depletion of plasma carotenoids during the inflammatory response might occur for several reasons. One possibility is that carotenoids might be incorporated into lymphoid tissues, where they act as immunomodulatory agents. In addition, carotenoids could be depleted from the plasma as antioxidants because of excess production of reactive species during the oxidative burst associated with inflammatory response (e.g. Walrand et al., 2005). However, it is also possible that changes of carotenoid metabolism during the acute-phase response are an indirect result of alterations in lipid metabolism without any carotenoid-specific regulation of tissue uptake (Koutsos et al., 2003). In any case, immune challenge with SRBC in captive greenfinches seemed to elicit much weaker inflammatory impact than LPS treatment in other species, which is perhaps not surprising given that SRBC, unlike LPS, might not stimulate robust innate immune responses (Klasing, 2004) (but see Eraud et al., 2005). This explanation would also be compatible with our previous results (Hõrak et al., 2003), where SRBC challenge caused only short-term elevation of markers of acute-phase response without any lasting effect on various plasma biochemicals, body mass or basal metabolic rate (BMR). However, costs arising from anti-SRBC response cannot be totally discounted because immune challenge with this antigen has been shown to elevate BMR in free-living great tits (*Parus major*) (Ots et al., 2001) and captive collared doves (*Streptopelia decaoto*) (Eraud et al., 2005). Moreover, production of antibody response against SRBC considerably reduced survival in incubating eiders (*Somateria mollissima*) (Hanssen et al., 2004).

We did not detect an effect of immune challenge on two different biomarkers of total antioxidant protection (TAS and AOP). This result contradicts our expectations based on the assumption that possible immunopathological damages, accompanying immune response, result from excess production of reactive species (e.g. Knight, 2000). For instance, inflammatory response accompanying strenuous exercise can reduce TAS (e.g. Ficicilar et al., 2003), indicating that in some situations, reduced TAS levels reflect hosts' inability to deal effectively with increased free radical load. However, an increase in TAS following exercise-induced oxidative stress has also been demonstrated (e.g. Vider et al., 2001), suggesting that, in some situations, increased TAS levels reflect compensatory enhancement of antioxidant defences. However, none of these changes could be associated with immune system activation in our study, which suggests that alterations of the total plasma antioxidant potential do not play any important role in forming the costs of SRBC-induced immune activation. Similarly, Alonso-Alvarez et al. did not find an effect of LPS injection on whole-blood antioxidant protection in zebra finches (Alonso-Alvarez et al., 2004).

Effects of carotenoid supplementation

Despite the pronounced effect of lutein supplementation on plasma carotenoid levels, we did not find any indication that this supplementation had affected the indices of immunocompetence measured in our study. Birds with almost depleted levels of carotenoids were capable of mounting similar primary and secondary anti-SRBC antibody titres and swelling responses to PHA as those circulating relatively high physiological doses of carotenoids. In this respect, our findings diverge from those of mammal studies (Jyonouchi et al., 1994; Kim et al., 2000a; Kim et al., 2000b), where lutein

supplementation has been shown to enhance immunoglobulin (IgG) production and/or T-cell proliferation. In chickens, lutein supplementation affected PHA-stimulated lymphocyte proliferation (Selvaraj et al., 2006), whereas no effect of antibody production against KLH (Selvaraj et al., 2006) or Newcastle disease virus (Haq et al., 1996) was detected. In moorhen (*Gallinula chloropus*) chicks, canthaxanthin supplementation enhanced PHA-response (Fenoglio et al., 2002). In passerines, two studies on captive zebra finches have found that lutein supplementation enhances PHA response (Blount et al., 2003; McGraw and Ardia, 2003); the latter study also found that carotenoid-supplemented birds mounted stronger antibody titres against SRBC. However, a range of dietary xanthophyll concentrations administered to male American goldfinches had no effect on several aspects of immunity and disease resistance (Navara and Hill, 2003).

One possible explanation for the discrepancy between these results might relate to the use of carotenoids for signalling purposes. Unlike greenfinches and goldfinches, whose carotenoid-based plumage coloration only signals their condition during moult, zebra finches can use dietary carotenoids to signal their current health status by flexibly changing their beak colour (a sexually selected signal) in response to circulating carotenoid levels (Blount et al., 2003). One might thus speculate that immunostimulatory effects of carotenoids are more likely to emerge in species possessing flexible (e.g. beaks and bare parts) rather than relatively static (e.g. plumage) carotenoid-based ornaments.

Independent of immune system activation, lutein supplementation affected fat deposition patterns as indicated by the significant increase in plasma triglyceride levels among supplemented birds (Fig. 2C). To our knowledge, such a phenomenon has not been previously described in an abundant carotenoid literature. We can exclude the possibility that carotenoid supplementation might have alleviated the coccidian-induced intestinal damage, known to suppress plasma triglyceride levels (Hõrak et al., 2004), because none of our treatments interfered with the dynamics of infection. Carotenoids are transported in blood by lipoproteins (mainly VLDL) (e.g. McGraw and Parker, 2006; McGraw et al., 2005b), which constitute the major part of plasma triglycerides. Thus, it seems that dietary lutein supplementation eventually leads to increased VLDL assembly in the liver, which inevitably results in elevation of circulating triglycerides as a by-product.

Antioxidant protection and carotenoids

Under the hypothesis that carotenoids significantly contribute to antioxidative protection, we predicted that individual plasma carotenoid levels correlate positively with measures of total antioxidativity. However, no such correlations emerged. We are confident that this lack of correlations cannot be ascribed to measurement techniques, because our estimates of total antioxidativity, obtained by two different assays, were highly correlated (Fig. 3). In line with our results, serum carotenoid concentration did not correlate with measures of antioxidant protection and serum concentration of reactive oxygen metabolites in a recent study of kestrel (*Falco tinnunculus*) nestlings (Costantini et al., 2006). Similarly, lutein supplementation to adult captive zebra finches had no direct effect on resistance of erythrocytes to oxidative lysis (Alonso-Alvarez et al., 2004). One possible explanation for these results would be that local actions of carotenoids in specific tissues are not reflected at the systemic level, so that plasma total antioxidant capacity is not affected. Such an explanation would be consistent with the results of Woodall et al., who demonstrated that despite the significant effect of zeaxanthin supplementation on plasma carotenoid levels in chicken, plasma lipid peroxidation was not affected by the treatments (Woodall et al., 1996). However, the lipid peroxidation in the liver was reduced by 78% when compared with the unsupplemented controls. Lack of correlation between plasma carotenoids and indices of total antioxidativity can also be reconciled with the results of an extensive meta-analysis of clinical studies of oxidative stress (Dotan et al., 2004), revealing that only under severe pathological conditions do all the indices of oxidative stress correlate with each other. However, at present, we cannot also totally exclude the alternative explanation, namely that systemic antioxidant properties of carotenoids in birds (except well-established protective effects on embryos and hatchlings) might not appear as important as previously thought, at least in situations where redox homeostasis is not threatened (see also Hartley and Kennedy, 2004).

In conclusion, our study found some evidence regarding the costs of humoral immune challenge and that some of these (reduced mass gain) can be alleviated by carotenoid supplementation. However, we did not find that immune challenge had induced any pathological damages that could be ascribed to oxidative stress. Carotenoid supplementation inclined birds to fattening, indicating that lutein interfered with lipid metabolism. Thus, although our results support the hypotheses of biological importance of carotenoids, they also exemplify the overwhelming complexity of their integrated ecophysiological functions.

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Notes and Comments

Do Dietary Antioxidants Alleviate the Cost of Immune Activation? An Experiment with Greenfinches

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ABSTRACT: Reactive oxygen and nitrogen species produced by metabolism and immune defenses can cause extensive damage to biomolecules. To counteract this damage, organisms rely on exogenous and endogenous antioxidants, although their relative importance in maintaining redox balance is unclear. We supplemented captive greenfinches with dietary antioxidants—carotenoids and vitamin E—and injected them with an inflammatory agent, phytohemagglutinin. Compared to controls, immune-challenged birds circulated more lipid peroxidation products but also increased total plasma antioxidativity. Carotenoid (but not vitamin E) supplementation generally reduced lipid peroxidation, but this did not compensate for the effects of immune activation. Levels of an endogenous antioxidant—uric acid—strongly contributed to plasma antioxidativity. We found no evidence that dietary antioxidants are immunostimulatory. These results demonstrate the antioxidant function of carotenoids in birds and show that simultaneous assessment of oxidative stress-driven damage, antioxidant barrier, and individual antioxidants is critical for explaining the potential costs of immune system activation.

Keywords: immune challenge, malondialdehyde (MDA), phytohemagglutinin, plasma carotenoids, total antioxidant capacity, uric acid.

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Cellular metabolism generates reactive oxygen and nitrogen species (RONS) that can damage lipids, proteins, and nucleic acids (Halliwell and Gutteridge 1999). To counteract harmful effects of excess RONS, organisms rely on a complex antioxidant network that includes endogenously produced enzymes and low-molecular-weight compounds and exogenous, food-derived antioxidants. The situation where RONS overwhelm the antioxidant defenses is defined as oxidative stress (OxS; Halliwell and Gutteridge 1999). Understanding how organisms adjust their antioxidant defenses to cope with OxS is important for several major areas of evolutionary ecology. It is believed that OxS represents the main proximate mechanism driving the aging process (Beckman and Ames 1998), and diversity of antioxidant defense systems in animals may explain evolutionary patterns of metabolic rates (reviewed by Perez-Campo et al. [1998]). Also, because RONS generation is accelerated by a high work load, OxS may increase with reproductive effort (Alonso-Alvarez et al. 2004*b*; Wiersma et al. 2004). The concept of OxS is also relevant to host-parasite coevolutionary research because production of RONS in the course of an immune response is an important source of immunopathology, which contributes to the costs of immunity (von Schantz et al. 1999; Splettstoesser and Schuff-Werner 2002). Finally, many types of animal pigments (McGraw 2005), particularly carotenoid-based ones (Lozano 1994), possess antioxidative properties and thus have the potential to honestly reveal the ability of their bearers to cope with OxS (von Schantz et al. 1999). Recently, however, the antioxidant role of carotenoids has been debated by Hartley and Kennedy (2004), who proposed that carotenoid-based traits might instead signal the availability of other nonpigmentary antioxidants that protect carotenoids from oxidation and thereby make them available for sexual displays.

Despite the prominence of the topic, attempts by animal ecologists to assess the importance of OxS and antioxidant defenses in vertebrate models have progressed slowly. Since the publication of a seminal paper by von Schantz et al. in 1999, only a handful of studies have managed to address

these issues. The few successful examples in birds include demonstrations that embryos are protected from oxidative damage by maternally derived carotenoids (Blount et al. 2002*a*, 2002*b*; McGraw et al. 2005*a*) and that reduced antioxidant protection is associated with increased reproductive effort (Alonso-Alvarez et al. 2004*b*; Wiersma et al. 2004; Bertrand et al. 2006*a*). On the other hand, attempts to demonstrate the antioxidant function of carotenoids in the postembryonic stage have been unsuccessful (Alonso-Alvarez et al. 2004*a*; Isaksson et al. 2005; Costantini et al. 2006; Hõrak et al. 2006; Tummeleht et al. 2006; Cohen et al. 2007). Studies aimed at assessing whether immune system activation leads to oxidative damage have yielded contradictory results. Bertrand et al. (2006*b*) showed that immune challenge of zebra finches (*Taeniopygia guttata*) with bacterial lipopolysaccharide (LPS) reduced the antioxidant barrier of blood. In the same model system, Alonso-Alvarez et al. (2004*a*) did not detect the effect of immune challenge on the blood antioxidant barrier, although immune activation diverted carotenoids from plasma. Contrary to this, Costantini and Dell'Omo (2006) found that in the nestlings of Eurasian kestrels (*Falco tinnunculus*), immune challenge with phytohemagglutinin (PHA) resulted in increased circulating levels of carotenoids, in addition to increasing the levels of reactive oxygen metabolites and decreasing the serum antioxidant barrier. Hõrak et al. (2006) did not detect any effects of repeated humoral immune challenge with sheep red blood cells on the plasma antioxidant barrier or carotenoid levels in captive greenfinches (*Carduelis chloris*).

The diversity of outcomes of those experiments can probably be partly ascribed to different study species, antigens, and experimental conditions. However, it is also possible that the difficulties in establishing the costs of immune activation relate to assessment methods of antioxidant protection and interpretations of results. For instance, the antioxidant barriers measured in the abovementioned studies are based on the capacity of the whole blood (or plasma or serum) to resist controlled free-radical attack ex vivo. These measurements, which are based on the cumulative action of all the antioxidants in the sample, may reflect the dynamics of protective mechanisms aimed at maintaining redox balance. This means that an increased antioxidant capacity of blood may not be a sign of optimal health condition if it reflects a compensatory response to increased oxidative stress. Similarly, a decrease in the antioxidant barrier may not necessarily signal deteriorated condition if it occurs in response to decreased production of reactive species (e.g., Prior and Cao 1999). Such problems of interpretation, for instance, frequently occur in sports medicine, where exercise-induced inflammatory responses elicit compensatory increases in the antioxidant barrier (e.g., Vider et al. 2001), so that often, the only evidence that oxidative stress has occurred may be the upregulation of antioxidant defense systems (Halliwell and Gutteridge 1999). Because of such complications, assessments of antioxidant protection should preferably also include estimates of oxidative stress-induced damage, such as lipid or protein peroxidation products (e.g., Prior and Cao 1999), which have been measured in only a few ecological studies (Blount et al. 2002*a*, 2002*b*; McGraw et al. 2005*a*).

The aims of this study are to ascertain whether activation of the immune system exacts oxidative costs in a passerine bird and to examine to what extent exogenous dietary antioxidants mitigate potential damage from RONS. For this purpose, captive greenfinches were first supplemented with combinations of lutein and vitamin E, two exogenous antioxidants that can be acquired in the diet, and subsequently either injected with saline or immune-challenged with a plant lectin PHA. Specifically, we examined four hypotheses. First, we hypothesized that activation of the immune system will lead to oxidative damage in tissue, as measured by increased lipid peroxidation, and a decline in plasma carotenoid levels and total antioxidant protection. Alternatively, an increase in plasma carotenoids and/or antioxidant protection would indicate that immune challenge promotes mobilization of antioxidant defenses. Second, we predicted that exogenous supplementation of carotenoids and vitamin E would alleviate the oxidative costs of immune system activation by reducing lipid peroxidation and increasing antioxidative protection. Third, we hypothesized that birds receiving antioxidant supplementation would mount stronger immune responses than birds receiving no supplementation, as documented in previous studies. Finally, we tested for correlations between measures of lipid peroxidation, total antioxidant protection, carotenoids, and an endogenous antioxidant, uric acid, to examine the relationships that promote redox balance and alleviation of oxidative stress. We predicted that exogenous and endogenous levels of antioxidants would be positively correlated with each other and negatively correlated with oxidative stress–induced damage after immune challenge.

Methods

Greenfinches weigh about 30 g and are sexually dichromatic, gregarious, granivorous passerines native to the western Palearctic region. Carotenoid-based plumage coloration in males is sexually selected (Eley 1991) and affected negatively by intestinal, viral, and hematozoan infections (reviewed by Hõrak et al. [2004]). Male greenfinches ($n = 94$) were caught in mist nets in the Sõrve Bird Observatory on the island of Saaremaa (57°55′N; 22°03′E) on January 22, 2006 (day 1). Birds were trans-

ported to Tartu and housed indoors (two identical rooms) in individual cages (27 cm × 51 cm × 55 cm) with sand bedding. Average temperature in the aviary during the experiment was 16.0° ± 1.6°C (SD), and average humidity was 51.5% ± 2.4% (SD). The birds were supplied ad lib. with sunflower seeds and filtered tap water. Birds were held on the natural day-length cycle. All blood samples were collected before the lights were turned on, in order to obtain the values of biochemical parameters characteristic of the state of overnight fast. Before each blood sampling, body mass of birds was recorded with a precision of 0.1 g. Blood sampling (by two persons) started 2.5 h before sunrise and lasted up to 4 h; "night" was artificially extended on blood-sampling days. Birds were brought from the aviary rooms into the bleeding room one at time, and maximum effort was made to avoid waking up the rest of birds (by keeping silent and using flashlights with small focus). Order of bleeding did not affect systematically any of the studied parameters (app. B in the online edition of the *American Naturalist*). Sampling of a single bird lasted about 4–5 min. Birds were released into their natural habitat on April 1 (day 72). The study was conducted under a license from the Estonian Ministry of the Environment.

Experimental Time Line

After transportation to Tartu, birds were allowed a 17-day acclimatization period (days 3–19). Birds were divided into six treatment groups (15–16 birds in each). These groups were set to have similar average body mass at capture and age composition (eight or nine first-year and seven older birds in each group). On the morning of day 20, preexperimental blood samples were collected, and subjects in each treatment group (except controls) started to receive different antioxidant supplements, as indicated in figure 1.

Birds were divided into the following treatment groups: (1) Carotenoid birds received 12 μg/mL carotenoid solution on alternate days, for a total of 13 days of antioxidant supplementation. (2) High–vitamin E birds received 500 μg/mL tocopherol solution on alternate days (13 days). (3) Low–vitamin E birds received 250 μg/mL tocopherol solution on alternate days (13 days). (4) Carotenoid + high–vitamin E birds received 12 μg/mL carotenoid solution and 500 μg/mL tocopherol solution on alternate days, a total of 26 days of supplementation. (5) Carotenoid + low–vitamin E birds received 12 μg/mL carotenoid solution and 250 μg/mL tocopherol solution on alternate days (26 days). (6) Control birds received just filtered tap water. Carotenoid supplementation consisted of lutein and zeaxanthin (20 : 1, w/w), prepared from OroGlo liquid solution of 11 g/kg xanthophyll activity (Kemin AgriFoods Europe, Herentals, Belgium). Vitamin E supplementation consisted of a mixture of 20 mg/mL d-α-tocopherol, 12 mg/mL d-γ-tocopherol, 4 mg/mL d-β-tocopherol + d-δ-tocopherol, and 2 mg/mL total tocotrienols, prepared from water-soluble vitamin E solution Aqua-E (Yasoo Health, Johnson City, TN). Those solutions were freshly prepared each evening using filtered (Brita Classic; BRITA, Taunusstein, Germany) tap water and were provided in 50-mL doses in opaque dispensers in order to avoid oxidation of carotenoids and vitamin E. Carotenoid dose was determined on the basis of previous work (Hõrak et al. 2006). The high dose of vitamin E was set to approximately double the content of tocopherols (mainly α-tocopherol) contained in sunflower seeds (Yoshida et al. 2002); the low dose of vitamin E was 50% of the high dose.

On day 34 (14 days after antioxidant treatments started), a second blood sample was collected (*mid exp.* in fig. 2). Subsequently, all the birds were subjected to 4-day anticoccidian treatment with Intracox Oral (Interchemie, Castenray, The Netherlands). The birds received 2 mL/L of the solution containing 25 mg/L toltrazuril in their drinking water. Antioxidant supplementation was temporarily interrupted for that period. On day 47, all the birds were orally infected with ~2,000 coccidian (*Isospora*) oocysts, a mixed stock originating from multiple hosts. The prepatent period of *Isospora* is 6–7 days (Mehlhorn et al. 1986; U. Karu, unpublished data). Because we blood-sampled the birds 4 days after experimental infection, we could not determine whether the resistance to novel coccidian strains affected their condition. However, we could test whether the efficiency of anticoccidian treatment affected the studied variables. For this purpose, we compared the hematological parameters and PHA responses of birds whose parasites were more susceptible to anticoccidian treatment (i.e., with zero prevalence of coccidians at the postexperimental blood sampling) with those of birds who had naturally relapsed infections by that time.

On the evening of day 48 (between 1630 and 1840 hours), eight or nine birds from each treatment group were injected intradermally in the wing web with 0.2 mg of PHA in 0.04 mL of sterile isotonic saline. At the same time, the rest of the birds (seven or eight individuals per group) were injected with saline. Wing web thickness was measured before injection, and the swelling response was measured 24 h later. Average increase in wing web thickness was 0.73 ± 0.31 mm ($n = 50$) for immune-challenged and 0.07 ± 0.11 mm ($n = 41$) for saline-injected birds ($t = 13.0$, $P < .00001$). Cutaneous hypersensitivity reaction resulting from PHA injection reflects the combined responses of T cells, cytokines, and inflammatory cells (Martin et al. 2006). We followed the simplified pro-

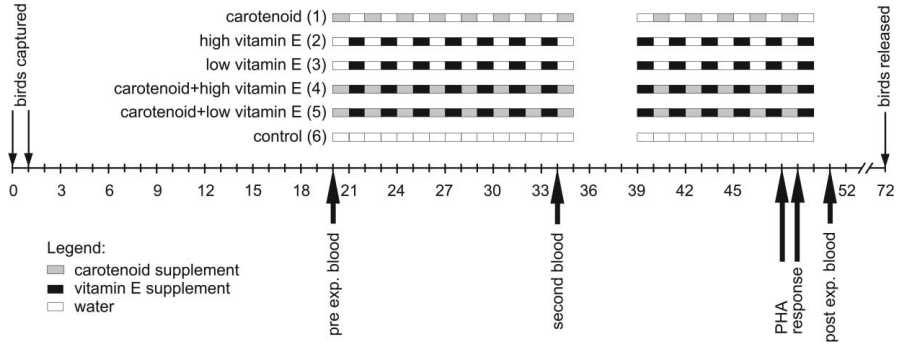

Figure 1: Course of the experiment. Day 0 = January 20. The break in antioxidant supplementation (days 35–39) occurred because of anticoccidian treatment of all birds.

tocol (Smits et al. 1999) as described in detail by Saks et al. (2003).

On the morning of day 51 (3 days after immune challenge), a third blood sample (time point *post exp.* in fig. 2) was collected. In all cases, 100–300 μL of blood was drawn from brachial or tarsal veins. After centrifugation, plasma was stored at −75°C until analyzed. During the few hours between blood collection and centrifugation, samples were maintained in refrigerator at 4°C. Lipid peroxidation products were determined only from the third blood sample (postexperimental); the rest of analyses were conducted for all three sampling points.

Chemical Analyses

Antioxidants. Concentrations of carotenoids were determined spectrophotometrically using acetone-resistant microtiter plates, as described by Hõrak et al. (2006). In this process, 150 μL of acetone was added to 15 μL of plasma and centrifuged for 10 min at 16,800 *g*. Absorbance of supernatant was measured at 449 nm. Concentration of uric acid was determined from 5-μL plasma samples by enzymatic colorimetric test with lipid clearing factor (uric acid liquicolor, HUMAN, Wiesbaden, Germany).

Total Antioxidant Potential. The capacity of fluids to inhibit redox reaction induced by free radicals was used to assess the total antioxidant potential (AOP) of plasma. Reduced AOP levels may reflect hosts' inability to deal with increased free-radical load. Increased AOP levels may represent a compensatory enhancement of antioxidant defenses (e.g., Prior and Cao 1999). The AOP was estimated using the BIOXYTECH AOP-490 assay (Oxis Research, Portland, OR), which is based on the reduction of Cu^{++} to Cu^{+} by the combined action of all antioxidants present in a sample. A chromogenic reagent, bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline), selectively forms a 2 : 1 complex with Cu^{+} that has a maximum absorbance at 490 nm. The color change of plasma incubated with reagent containing Cu^{++} and chromogen for 3 min at room temperature is measured. A standard of known uric acid concentration was used to create the calibration curve, so that the results are quantified in millimole-per-milliliter uric acid equivalents. The assay was adapted for small (5-μL) plasma samples. Previous research (Hõrak et al. 2006; Tummeleht et al. 2006) has

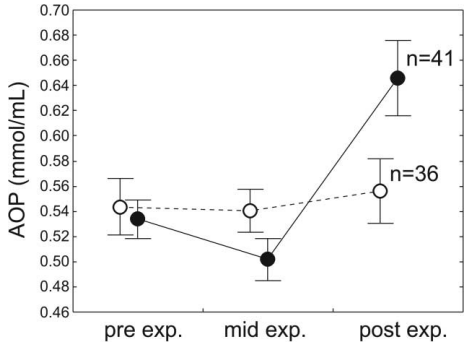

Figure 2: Dynamics of plasma antioxidant potential (AOP) among immune-challenged (*filled circles*) and control (*open circles*) birds. Error bars represent SE.

shown that this method is precise and that the measurements of antioxidativity obtained by the AOP kit strongly and linearly correlate with estimates obtained by the trolox equivalent antioxidant capacity assay (Randox TAS kit, Randox, Crumlin, United Kingdom). All spectrophotometric analyses (except lipid peroxidation) were performed with Tecan microplate reader (Sunrise, Tecan Austria, Grödig/Salzburg, Austria).

Lipid Peroxidation. Lipid peroxidation (LPO) is a well-established mechanism of cellular injury used to indicate OxS in cells and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds, including reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals (HAEs) upon decomposition. The LPO-586 assay (Oxis Research) is based on the reaction of a chromogenic agent, N-methyl-2-phenylindole, with MDA and HAEs at 45°C. One molecule of MDA or HAE reacts with two molecules of reagent to yield a stable chromophore with maximal absorbance at 586 nm.

After separation of plasma, 1 μL of 0.125 M butylated hydroxytoluene (BHT) solution in acetonitrile was added to a 100-μL plasma sample in order to preserve it from oxidation. The assay was performed according to the kit instructions, which were adapted for 50-μL plasma samples by reducing the amounts of all required chemicals four times. Absorbance was measured against sample blank containing 50 μL plasma and a mixture of acetonitrile and diluent. The standard curve was prepared from the acetal (tetramethoxypropane [TMOP]; stock solution provided with the kit), which is hydrolyzed during the acid incubation step at 45°C, generating MDA. The results are expressed as nanomoles of MDA per milliliter of plasma.

Statistics

Effects of experimental treatments on the dynamics of AOP, uric acid, and body mass were analyzed by repeated-measures ANOVAs. Carotenoid and vitamin E treatments and PHA versus saline injections were considered as factors with two, three, and two levels, respectively. The blood-sampling event was a within-individual repeated measure termed "time" (with three levels: preexperiment, mid-experiment, and postexperiment; fig. 1). We started examination of full models with interaction terms between all factors and time, dropping nonsignificant effects one at a time. In the final models, only significant main effects and interactions were retained. The effects of treatments were assumed to be revealed by significant time × treatment interaction terms. Assumptions for this analysis (normality, homogeneity of variances, and sphericity) were met for these variables (for AOP and uric acid, this required ln transformation). Because carotenoid concentrations were not normally and homogenously distributed, we could not apply repeated-measures ANOVA for testing the treatment effects. Therefore, individual changes within treatment groups were tested with Friedman's ANOVA and between-treatment differences with *U*-tests (table 1). The LPO products were measured only once during the experiment (after PHA challenge). Treatment effects on LPO and swelling response to PHA were tested in ANOVA, starting with full models of all factors and interactions. The PHA response was normally distributed, and distribution of LPO values became normal after ln transformation. Assumption of homogeneity of variances was met for both analyses. All initial and final models retained in the analyses are presented in appendix A in the online edition of the *American Naturalist*. Relationships between individual antioxidants and LPO, AOP, and swelling response were assessed on the basis of Spearman rank correlations. Because vitamin E supplementation did not affect any of the studied parameters (app. A), data were pooled over experimental groups 1, 4, and 5 (carotenoid supplemented) and over groups 2, 3, and 6 (unsupplemented with carotenoids) for the final analyses of the effects of immune challenge and carotenoid supplementation. Sample sizes differ between some analyses because of our inability to

Table 1: Average plasma carotenoid concentrations (μg/mL) in different treatment groups and *P* values and *Z* statistics for between-group comparisons (Mann-Whitney *U*-tests)

	No carotenoid supplementation						Carotenoid supplementation							
	PHA		Saline		*P*		PHA		Saline		*P*		Difference[a]	
Time	Mean ± SD	*n*	Mean ± SD	*n*	diff.	*Z*	Mean ± SD	*n*	Mean ± SD	*n*	diff.	*Z*	*P* diff.	*Z*
Preexp.	8.5 ± 8.7	24	8.1 ± 6.6	20	.777	−.28	10.2 ± 7.6	24	7.3 ± 6.0	20	.199	1.29	.310	1.02
Mid-exp.	3.4 ± 4.8	25	3.3 ± 3.2	19	.619	−.50	17.8 ± 8.3	21	15.4 ± 11.3	19	.140	1.48	<.00001	6.60
Postexp.	1.6 ± 2.6	24	2.7 ± 3.3	18	.322	−.99	10.6 ± 5.7	22	9.7 ± 9.9	18	.289	1.06	<.00001	5.83

Note: "Preexp.," "Mid-exp.," and "Postexp." stand for the preexperimental, mid-experimental, and postexperimental blood samples, respectively. Individual carotenoid levels changed during the experiment both among unsupplemented ($\chi^2 = 43.45$, $P < .00001$, $n = 40$) and carotenoid-supplemented birds ($\chi^2 = 34.37$, $P < .00001$, $n = 38$; Friedman ANOVA).

[a] Difference between unsupplemented and carotenoid-supplemented groups.

collect sufficient amount of blood from all the birds. Average trait values ± SD are presented. Age (first year vs. older) did not affect any of the studied parameters (app. B).

Results

Three days after injection, immune-challenged birds circulated 19% higher levels of lipid peroxidation (LPO) end products in their plasma than saline-injected birds (PHA-injected: 1.51 ± 0.71 nmol MDA/mL plasma, $n = 41$; saline-injected: 1.23 ± 0.68 nmol MDA/mL, $n = 33$; $t = 2$, $P = .049$; untransformed means ± SD, *t*-test on ln-transformed values). Plasma antioxidant potential (AOP) of immune-challenged birds increased significantly after PHA injection compared to that of nonchallenged birds, whose AOP remained stable during the study period (fig. 2; $F = 7.7$, df = 2, 150, $P = .0007$ for time × immune challenge interaction term in repeated-measures ANOVA with main effects of immune challenge [$F = 0.4$, df = 1, 75, $P = .552$] and time [$F = 9.4$, df = 2, 150, $P = .0001$]). Immune challenge did not affect the dynamics of plasma carotenoid levels (table 1). Immune challenge did not affect the dynamics of plasma uric acid levels ($F = 2.6$, df = 2, 112, $P = .076$ for time × immune challenge interaction term in repeated-measures ANOVA with main effects of immune challenge [$F = 0.2$, df = 1, 56, $P = .688$] and time [$F = 6.6$, df = 2, 112, $P = .001$]). Vitamin E treatment did not affect LPO ($F = 0.4$, df = 2, 62, $P = .690$) or dynamics of AOP ($F = 0.3$, df = 4, 130, $P = .864$), plasma carotenoids at the end of the experiment ($H = 0.05$, $P = .977$, $n = 85$; Kruskal-Wallis ANOVA), or uric acid ($F = 1.1$, df = 4, 92, $P = .378$); see appendix A.

Carotenoid supplementation significantly increased plasma carotenoid levels, while among unsupplemented birds, plasma carotenoids declined throughout the study (table 1). Carotenoid supplementation significantly reduced LPO (MDA levels in supplemented birds: 1.21 ± 0.62 nmol/mL, $n = 39$; in unsupplemented birds: 1.59 ± 0.75 nmol/mL, $n = 37$; app. A). The effect of carotenoid supplementation on LPO remained significant in ANOVA models adjusting for the effect of immune challenge on LPO ($F = 7.3$, df = 1, 71, $P = .008$). However, carotenoid treatment did not significantly alleviate the effect of immune challenge on LPO, as indicated by the nonsignificant interaction term between immune challenge and carotenoid supplementation (fig. 3; $F = 0.11$, df = 1, 70, $P = .735$) in a model with main effects of immune challenge ($F = 3.4$, df = 1, 70, $P = .068$) and carotenoid treatment ($F = 7.0$, df = 1, 70, $P = .010$). As shown above, carotenoid supplementation did not affect the dynamics of AOP in the model adjusting for the effects of immune challenge on AOP dynamics. Neither did we find any evidence that carotenoid supplementation had modulated the effect of immune challenge on the dynamics of AOP ($F = 1.8$, df = 2, 130, $P = .166$ for a three-way interaction between time × immune challenge × carotenoid supplementation terms in repeated-measures ANOVA). Carotenoid supplementation did not affect the dynamics of uric acid ($F = 1.2$, df = 2, 92, $P = .307$). Experimental treatments did not affect body mass dynamics (app. A); neither did body mass correlate significantly with any of the measured biochemical parameters or swelling response to PHA.

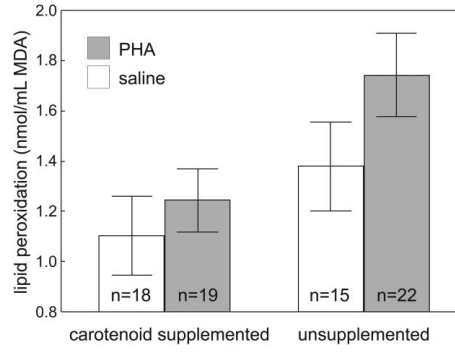

Figure 3: Effect of carotenoid supplementation and immune challenge on lipid peroxidation, measured as plasma malondialdehyde (MDA) concentration. Error bars represent SE. See "Results" for ANOVA statistics.

Antioxidant supplementation did not affect the magnitude of the swelling response to PHA injection ($F = 0$, df = 1, 44, $P = .980$ for carotenoids; $F = 0.3$, df = 2, 44, $P = .70$ for vitamin E; and $F = 0.7$, df = 2, 44, $P = .50$ for carotenoid × vitamin E interaction). Magnitude of swelling response to PHA did not correlate with concentrations of lipid peroxidation products, carotenoids, or uric acid measured 3 days after injection ($r_s = 0$–0.1, $P = .7$–$.9$, $n = 32$–46).

At the end of the experiment, the antioxidant barrier increased with increasing plasma uric acid levels (AOP vs. uric acid: $r_s = 0.81$, $P < .0001$, $n = 65$; fig. 4) and LPO (AOP vs. LPO: $r_s = 0.42$, $P = .0003$, $n = 72$; fig. 4). Lipid peroxidation also correlated positively with plasma uric acid levels (uric acid vs. LPO: $r_s = 0.37$, $P = .005$, $n = 55$; fig. 4). Plasma carotenoid levels were not related to antioxidant barrier, LPO, or uric acid levels ($r_s = -0.2$ to 0.2, $P = .06$–$.4$, $n = 64$–82). Relapse of coccidian infection after medication was not related to any of the physiological parameters measured at the postexperiment blood sampling or to swelling response to PHA (app. B).

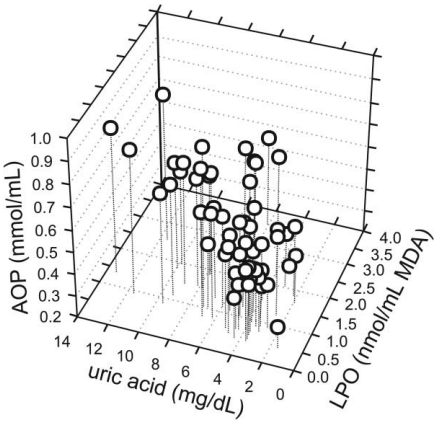

Figure 4: Relationships between lipid peroxidation (*LPO* measured as plasma malondialdehyde [MDA] concentration), plasma uric acid concentration, and antioxidant potential. See "Results" for the correlation statistics.

Discussion

This study clearly showed that activation of the immune system in greenfinches increased lipid peroxidation and elevated plasma antioxidant potential. Carotenoid- (but not vitamin E-) supplemented birds circulated lower levels of lipid peroxidation products, but the effect of supplementation was not strong enough to reduce lipid peroxidation caused by immune challenge. Levels of an endogenous antioxidant—uric acid—strongly and positively correlated with total plasma antioxidant potential. Finally, supplementation with dietary carotenoids or vitamin E failed to stimulate immune function in greenfinches.

Lipid peroxidation involves a chain reaction whereby free radicals remove electrons from the lipids of membranes surrounding cells and organelles such as mitochondria, lysosomes, and peroxisomes (Halliwell and Gutteridge 1999). It most often affects polyunsaturated fatty acids (PUFAs), which are responsible for maintenance of physiologically important membrane properties, including fluidity and permeability. Peroxidation products of PUFAs, such as MDA and alkenals, are also mutagenic and cytotoxic and can damage membrane proteins (Halliwell and Gutteridge 1999). To our knowledge, this study presents the first direct evidence in a wild animal species that immune system activation by a nonparasitic foreign antigen leads to lipid peroxidation. Notably, a similar study in kestrel nestlings found that PHA challenge increased the serum concentrations of reactive oxygen metabolites that are able to propagate the chain reactions that may eventually lead to lipid peroxidation (Costantini and Dell'Omo 2006).

Phytohemagglutinin (PHA) is a plant lectin derived from the red kidney bean (*Phaseolus vulgaris*) where it is believed to serve as a defense against herbivory. Injection with PHA induces a complicated immunological cascade beginning with proliferation of (mainly T) lymphocytes and secretion of proinflammatory cytokines that recruit and activate effector cells and phagocytes such as basophils, heterophils, and macrophages (Martin et al. 2006). Eventually, infiltration and additional cytokine secretion by these phagocytes is believed to induce the local swelling response (Martin et al. 2006). Besides the local leukocyte infiltration, PHA injection in birds can also elevate the concentration of circulating heterophils in the peripheral blood for as long as 1 week after challenge (Hõrak et al. 2000), which points to the induction of systemic inflammation. Heterophils involved in this process represent the first line of immune defense in terms of ingestion and destruction of potential pathogens. During particle ingestion, they produce large amounts of RONS, which, in addition to the pathogens, can damage the phagocytes themselves and other exposed cells (Splettstoesser and Schuff-Werner 2002). We consider such inflammation-induced damage as the most parsimonious explanation for the PHA-induced lipid peroxidation observed in our experiment. Such an interpretation would be consistent with evidence of occurrence of lipid peroxidation in inflammations associated with infectious and degenerative diseases (Romero et al. 1998) or exhaustive physical exercise (e.g., Vider et al. 2001). Further research in this area would benefit from application of experimental immune suppression treatments. For instance, Alonso-Alvarez et al. (2007) found that experimental reduction of testosterone levels in zebra finches enhanced the resistance of erythrocytes to controlled free-radical attack and also increased the magnitude of swelling response to PHA.

In addition to increased lipid peroxidation in response to immune challenge, the other physiological processes accompanying this effect are of considerable interest. Approximately 60 h after immune challenge, the plasma antioxidant barrier of challenged birds increased 25% compared to the values measured 14 days before immunization. Among the saline-injected birds, the increase was only 4%. This result contrasts with the previous finding in kestrel nestlings (Costantini and Dell'Omo 2006), where immunostimulation with PHA caused a significant decrease of the serum antioxidant barrier 24 h after injection. A similar decrease in the whole-blood antioxidant barrier (measured as erythrocyte resistance to controlled free-radical attack) was observed in zebra finches 24 h after injection of bacterial lipopolysaccharide (LPS; Bertrand et

al. 2006*b*). On the other hand, Cohen et al. (2007) found no effect of LPS injection on plasma antioxidativity in chickens. Our results are more similar to those observed in exercise-induced inflammation, where plasma antioxidativity and/or antioxidant enzyme levels increased in parallel with lipid peroxidation (e.g., Vider et al. 2001; Tauler et al. 2006). These results exemplify the compensatory activation of endogenous antioxidant machinery in response to free-radical attack imposed by immune system activation.

Our result of a strong correlation between plasma antioxidativity and uric acid levels compares favorably with recent findings of Cohen et al. (2007), who demonstrated positive relationships between these variables in 92 wild bird species. The antioxidative properties of uric acid and its high concentrations in bird plasma have been suggested as one of the reasons for greater longevity of birds than mammals of similar size (Klandorf et al. 2001). The positive correlation between uric acid and lipid peroxidation (fig. 4) might indicate production of this antioxidant in response to increased lipid peroxidation. For instance, plasma uric acid has been shown to increase in parallel with lipid peroxidation in broiler chickens during chronic corticosterone exposure (Lin et al. 2004). It should be noted, however, that uric acid could not be considered a single major antioxidant contributing to the increased antioxidant barrier in response to immune challenge in our study, because the treatment effect on its temporal dynamics was not significant. Evidence in humans suggest that proteins and micromolecular antioxidants, such as ascorbate, polyphenols, and glutathione, can also importantly contribute to plasma antioxidativity (e.g., Erel 2004).

Despite the considerable variation in plasma carotenoid levels induced by dietary manipulation (table 1), plasma carotenoids did not correlate with plasma antioxidativity. This result is consistent with previous findings in captive greenfinches (Hõrak et al. 2006), breeding great tits (Tummeleht et al. 2006), and kestrel nestlings (Costantini and Dell'Omo 2006). On the other hand, in this study, carotenoid supplementation reduced lipid peroxidation by 24%. Although this effect was not strong enough to compensate for the increased LPO due to immune challenge, it indicates that the antioxidant function of carotenoids in avian models cannot be totally discounted, as suggested by Hartley and Kennedy (2004). Our results thus demonstrate the necessity of composite assessment of AOP and indices of oxidative stress-induced damage, because the antioxidative effect of carotenoid supplementation could be demonstrated only by measuring LPO products. We would have reached a different conclusion about the antioxidant function of carotenoids on the basis of AOP data alone.

The antioxidant function of carotenoids in birds has been well established in protection of embryos and hatchlings (Surai 2002; McGraw et al. 2005*a*). It remains to be shown whether these antioxidant properties are also of any ecological relevance in adult birds. In this context, it is important to note that the carotenoid manipulation in our study was relatively moderate. At the time of LPO measurement, supplemented birds circulated only about 10 μg/mL and unsupplemented birds about 2 μg/mL of carotenoids in their plasma. Indirect evidence suggests that the amounts circulated during molt in the wild may range up to at least 30 μg/mL (Karu et al. 2007). Thus, it appears feasible that plasma carotenoid levels (which are directly reflected in plumage coloration) may indeed signal the birds' ability to resist oxidative stress, especially if the energetic costs imposed by molt contribute to LPO. This contention is supported by evidence that nutritional limitation during molt (independent of carotenoid availability) reduces carotenoid ornament expression (Hill 2000; McGraw et al. 2005*b*). To prove that carotenoids exert any important physiological effect in birds as antioxidants, measurements of LPO, carotenoids, and other sources of antioxidant protection must be associated with components of fitness, preferably in the field. This study shows that measuring the antioxidant barrier alone is not sufficient to demonstrate the antioxidant function of carotenoids (see also Alonso-Alvarez et al. 2004*a*; Costantini et al. 2006).

Lipid peroxidation was not affected by vitamin E supplementation, although vitamin E is considered the main lipophilic antioxidant involved in membrane defense (Halliwell and Gutteridge 1999). Neither did carotenoid or vitamin E supplementation (separately or in combination) affect the strength of the swelling response to PHA. Immunostimulatory effects of vitamin E have been repeatedly demonstrated in poultry (Surai 2002). Possibly, the effects of vitamin E supplementation in our study could be masked by the high levels of this antioxidant in dietary sunflower seeds (Yoshida et al. 2002). To our knowledge, the only previous experiment of vitamin E supplementation in wild birds has been performed on nestling barn swallows (*Hirundo rustica*); no effect on PHA response was found (de Ayala et al. 2006).

Interest of animal ecologists in the immunostimulatory properties of carotenoids has emerged since publication of Lozano (1994). Since then, the associations between immune function and carotenoids have been implicated in at least eight avian species (reviewed by McGraw et al. [2006]); however, results demonstrating a lack of association have also started to accumulate (Navara and Hill 2003; McGraw and Ardia 2005; Hõrak et al. 2006; McGraw and Klasing 2006; McGraw et al. 2006). One reason for these inconsistencies may relate to the ways ecologists assess and interpret immunocompetence. For instance, the

PHA-induced skin swelling, which is arguably the most popular immune assay in the wild birds, results from the activation of both adaptive and innate components of the immune system (Martin et al. 2006), all of which might be differentially affected by different carotenoids. Furthermore, indices of immunocompetence obtained by measuring responses to novel antigens are notoriously difficult to interpret in the context of real parasite resistance (Adamo 2004). This also holds for the PHA-induced skin swelling that has been enhanced because of carotenoid supplementation in some but not in other studies (reviewed by McGraw et al. [2006]). In our greenfinch model, for instance, the strongest swelling response to PHA was produced by the individuals most susceptible to infection with novel coccidian strains (Saks et al. 2006). Such outcomes are perhaps expected, given that different compartments of immune system, orchestrated by secretion of cytokines by Th1 and Th2 lymphocytes, are in a cross-regulatory balance (reviewed by Graham et al. [2005]). This means that humoral Th2 responses may exert anti-inflammatory action by downregulating Th1 cell–mediated immunity and vice versa. Recently, it has been proposed that the immune system's constrained ability to achieve the optimal balance between those different arms of defense serves as a major evolutionary reason why natural selection has not eliminated immunopathology, which is implicated in the etiology of most diseases (Graham et al. 2005). Realizing that immune function is not a distinct and easily quantifiable entity is also necessary for understanding the costs of expression of signals based on carotenoids and other antioxidant pigments.

In conclusion, this study assessed for the first time a measure of oxidative stress–driven damage—lipid peroxidation—in a wild bird species in postembryonic stage. We found that this damage was induced by inflammatory immune challenge and reduced by carotenoid supplementation, although this reduction was not sufficient to compensate for the effect of immune system activation. These results demonstrate the importance of antioxidant function of carotenoids in birds. In addition, we found that immune challenge induced an increase in plasma total antioxidant capacity. This capacity was strongly correlated with the plasma levels of an endogenous antioxidant, uric acid, which is a simple end product of nitrogen metabolism. These results indicate that caution is required in interpreting the potential costs of immune system activation on the basis of assessment of blood antioxidant barrier alone. Identifying the mechanisms by which organisms cope with oxidative stress is necessary for understanding the major processes in important domains of evolutionary ecology. This study shows that these mechanisms cannot be properly elucidated without simultaneous assessment of oxidative damages, antioxidant barriers, and individual antioxidants.

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Male greenfinch (photograph by Arne Ader; http://www.loodusemees.ee).

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Appendix A. Effects of Experimental Treatments on Physiological Parameters of Greenfinches.

Appendix B. Effects of Age, Coccidian Infection Status, and Bleeding Time on Physiological Parameters of Greenfinches.

VI

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ORIGINAL ARTICLE

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Carotenoid-based plumage coloration is not affected by vitamin E supplementation in male greenfinches

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Abstract Carotenoid-based colours have become an important model of honest signalling as carotenoids are suggested to play vital roles in several physiological functions including antioxidants and immunostimulators, while they are also required for sexual displays. However, it has been recently suggested that carotenoid-based signals may be used mainly as reflectors of the systems that prevent their oxidation (mainly the amount of other non-pigmented antioxidants) rather than the antioxidative properties of carotenoids themselves. We tested this hypothesis by examining the effect of simultaneous supplementation of carotenoids and an uncoloured antioxidant—vitamin E—on the coloration of growing tail feathers in captive male greenfinches (*Carduelis chloris chloris* L.). While carotenoid supplementation enhanced the coloration of the feathers, manipulation of dietary vitamin E had no effect. Thus, our results do not support the idea that carotenoids are mainly used as indicators of the abundance of other antioxidants.

Keywords *Carduelis chloris* · Dietary antioxidants · Carotenoid supplementation

Introduction

Carotenoid-based signals have been the target of extensive research by animal ecologists since the recognition of their possible role in health maintenance and signalling. As the carotenoids must be acquired from food (Fox 1979) and are destroyed when used as antioxidants (Vershinin 1999), it is believed that their use as colorants in ornaments, such as feathers, skin and scales, may be traded off with their use in other functions (e.g. as immunostimulators or antioxidants). Therefore, it has been suggested that carotenoid-based visual characters enable individuals to convey honest information on their bearer's phenotypic and/or genetic quality to potential mates and opponents (e.g. Lozano 1994; Olson and Owens 1998; Møller et al. 2000; Hill and McGraw 2006). Only the highest quality individuals could allocate sufficient quantities of pigments to develop the most colorful signals without compromising the essential need of carotenoids for maintenance purposes at the same time.

In birds, the idea that carotenoid-based signals function as badges of individual quality has received considerable support from correlational and experimental studies that link the colour of such traits to foraging efficiency, disease status and immunocompetence (review in Hill and McGraw 2006). In this context the antioxidative nature of carotenoids is highly advocated (Lozano 1994; Olson and Owens 1998; von Schantz et al. 1999; Møller et al. 2000). Still, with few exceptions (Jaensch et al. 2001; Woodall et al. 1996), the antioxidant function of carotenoids in birds has been studied mainly in the context of embryo-protective maternal effects (Surai 2002; McGraw et al. 2005). Only a few studies (Alonso-Alvarez et al. 2004; Bertrand et al. 2006a; Costantini et al. 2006) have considered the relationships between carotenoids and general antioxidant defences in postembryonic stage. Furthermore, the idea that carotenoid-based ornaments are direct displays of their bearers antioxidative power via carotenoid compounds has been recently challenged by Hartley and Kennedy (2004). They argue that although carotenoids do exhibit antioxidant activity in vitro, this is not their primary biological role. Instead, they suggest that carotenoids may be used mainly as signals, revealing the amount of non-pigmented antioxidants (such as antioxidative enzymes and vitamins E and C), which are more important biological protectants against free-radical-mediated oxidative stress. Under this scenario, organisms rely on carotenoids to advertise their antioxidative potential as the presence of other (uncoloured) antioxidants would protect carotenoids from oxidative

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decoloration. To our knowledge, this hypothesis of Hartley and Kennedy (2004), that carotenoid-based coloration is affected by the availability of other antioxidants, has been tested only twice (Bertrand et al. 2006b; Pike et al. 2007). Supplementation with uncoloured antioxidants (melatonin and mixture of vitamins E and C, respectively) had an additive effect on the carotenoid-based ornamental coloration of the beaks in captive zebra finches (*Taeniopygia guttata*; Bertrand et al. 2006b) and nuptial skin patches in sticklebacks (*Gasterosteus aculeatus*; Pike et al. 2007). Still, whether these results can be generalised to other antioxidants and ornaments (such as feathers) remains untested. Feather ornaments are interesting in the context of signalling because once the carotenoids are deposited in such a metabolically inactive tissue, they become unavailable for other biological functions (Olson and Owens 1998). Hence, one might expect the carotenoid-based feather ornaments to be a particularly honest and long-term signal of individual ability to cope with oxidative stress.

We tested the hypothesis of Hartley and Kennedy by experimentally supplementing captive male greenfinches (*Carduelis chloris chloris* L.) with carotenoids and an uncoloured antioxidant, vitamin E. Greenfinches express several carotenoid-based plumage patches which are sexually selected (Eley 1991). The yellow colour of these feathers is based on canary xanthophylls A and B and their *cis* isomers (Saks et al. 2003), which are produced by interconversion of dietary lutein and zeaxanthin (Surai 2002). Intensity of the yellow coloration of male greenfinches reflects the amount of carotenoids deposited into feathers (Saks et al. 2003) and plasma carotenoid levels during feather growth (Karu et al. 2007). Carotenoid-based plumage coloration of male greenfinches is sensitive to intestinal (Hõrak et al. 2004b), viral (Lindström and Lundström 2000) and hematozoan (Merilä et al. 1999) infections.

Vitamin E is the main lipophilic antioxidant in animals, and it can only be procured from food. Due to the ability to scavenge peroxyl radicals and singlet oxygen, it is probably the most important agent in cell membrane defences as an inhibitor of the free-radical chain reaction of lipid peroxidation (Halliwell and Gutteridge 2004). Thus, the abundance of vitamin E can be expected to significantly affect the total antioxidative potential of an individual organism. Therefore, if the primary role of carotenoid-based signals is to reflect the levels of other antioxidants, then supplementary feeding with vitamin E should have a significant effect on the expression of carotenoid pigmented ornaments. Thus, we predicted that simultaneous supplementation of vitamin E and carotenoids would result in more colourful feathers than carotenoid supplementation alone.

Materials and methods

Ninety-four male greenfinches were caught in mist-nets in the Sõrve Bird Observatory on Saaremaa island (57°55′N; 22°03′E) between 20 (day 1 of the experiment) and 22 January 2006. Birds were transported to Tartu a day after capture and housed in individual indoor cages ($27 \times 51 \times 55$ cm) with sand bedding. Ad libitum sunflower seeds and filtered tap water were always available. Sunflower seeds contain on average 720 μg/g of tocopherols (Yoshida et al. 2002) and 1 μg/g of carotenoids (McGraw et al. 2001). During the experiment, the average temperature in the aviary was 16.0 ± 1.6 (SD)°C and the average humidity was 51.5 ± 2.4 (SD)%. Natural day-length cycle was maintained in the aviary throughout the experiment. Birds were released to their natural habitat on 1 April 2006 (day 72).

After transportation to Tartu, the birds were allowed a 17-day acclimation period (days 3–19). The birds were divided into six treatment groups (15–16 birds in each). These groups were set to have similar average body mass (group averages ranging from 28.1 to 28.8 g ± 1.7–2.0 SD) and age composition (8–9 first year and 7 older birds in each group). In the morning of day 20, the birds in each treatment group started to receive different antioxidant supplementations. Carotenoid dosage was based on our previous experience showing that supplementation with 10 μg/ml carotenoid solution during 33 days resulted in significant increase in plasma carotenoid levels (Hõrak et al. 2006) and lab-grown feather chroma (Karu et al. 2007) in supplemented birds as compared to controls. In this experiment, we increased the concentration of the solution (12 μg/ml), as the supplementation time was planned shorter (13 days). High dose of vitamin E was set to approximately double the content of tocopherols (mainly α-tocopherol) contained in sunflower seeds (Yoshida et al. 2002); low dose of vitamin E was 50% of high dose. The treatments were formed as follows (Fig. 1): (1) Carotenoid—birds received 12 μg/ml carotenoid solution on alternate days, altogether in 13 days. (2) High vitamin E—birds received 500 μg/ml tocopherol solution on alternate days, altogether in 13 days. (3) Low vitamin E—birds received 250 μg/ml tocopherol solution on alternate days, altogether in 13 days. (4) Carotenoid + high vitamin E—birds received 12 μg/ml carotenoid solution and 500 μg/ml tocopherol solution on alternate days, altogether 26 days of antioxidant supplementation. (5) Carotenoid + low vitamin E—birds received 12 μg/ml carotenoid solution and 250 μg/ml tocopherol solution on alternate days, altogether 26 days of antioxidant supplementation. (6) Control—birds received filtered tap water only. Carotenoid supplementation consisted of lutein and zeaxanthin (20:1, *w*/*w*), prepared from OroGlo liquid solution of 11 g/kg xanthophyll activity (Kemin AgriFoods Europe, Herentals, Belgium). Vitamin E supplementation was prepared from water-soluble vitamin E solution Aqua-E™ (Yasoo Health Inc., Johnson City, TN) which consisted of a mixture of 20 mg/ml *d*-α-tocopherol, 12 mg/ml *d*-γ-tocopherol, 4 mg/ml *d*-β-tocopherol + *d*-δ-tocopherol (respectively 56, 33 and 11% from total tocopherols) and 2 mg/ml total tocotrienols. In sunflower seeds, α-tocopherol is

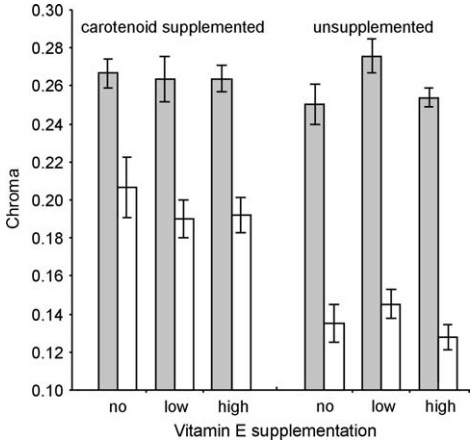

Fig. 1 Average (±SE) chroma values of wild-grown (*filled columns*) and replacement feathers grown during the experiment (*empty columns*) of different treatment groups ($n = 15$ in all groups)

predominant (over 90%), while γ- and β-tocopherol account for 6 and 1%, respectively (Yoshida et al. 2002). Similarily, in greenfinches, kept on sunflower seed diet, α-tocopherol formed 95% and γ-tocopherol 5% of total measured tocopherols (Hõrak et al. unpublished data). Supplement solutions were freshly prepared each evening using filtered (Brita® Classic; BRITA GmbH, Taunusstein, Germany) tap water and were provided in 50-ml doses in opaque dispensers in order to avoid oxidation of carotenoids and vitamin E. The effect of these supplementations on individual measures of antioxidants (carotenoids and uric acid), plasma antioxidant potential and lipid peroxidation products are discussed in Hõrak et al. 2007. We were unable to measure vitamin E hemoconcentrations due to technical reasons.

For the analysis of plumage colour, one right outermost (sixth) tail feather (wild-grown feathers) was collected from each individual during the 20th day of the experiment. Full-grown replacement feathers (lab-grown feathers) were collected from the same positions right before the birds were released (on the day 71). Collected feathers were stored in plastic bags in the dark. Colour measurements were performed as described in detail by Karu et al. (2007) and Hõrak et al. (2004b) using a spectrophotometer (Ocean Optics USB2000 with Ocean Optics DH2000 lamp). Plumage colour was characterised on the basis of chroma (see Endler 1990 for details) of the yellow parts of tail feathers. Measuring error of this method of plumage colour quantification is reasonably low as the repeatability (Lessells and Boag 1987) of three consecutive measurements of the same feather ranged from 0.73 to 0.88 (Saks et al. 2003).

Table 1 The effect of supplementary vitamin E and carotenoid feeding on feather chroma in repeated measures ANOVA

Effect	*df*	*F* value	*P* value
Univariate tests			
Carot.	1;84	33.96	< 0.00001
Vit. E	2;84	0.94	0.39
Carot. × Vit. E	2;84	2.23	0.11
Multivariate tests			
Time	1;84	305.45	< 0.00001
Time × Carot.	1;84	25.39	< 0.00001
Time × Vit. E	2;84	0.64	0.53
Time × Carot. × Vit. E	2;84	0.006	0.99

"Carot." and "Vit. E" stand for carotenoid supplementation and vitamin-E supplementation, respectively. "Time" represents the difference in chroma of wild-grown versus lab-grown feathers within individuals

The effect of the treatments on the feather chroma were examined with repeated-measures ANOVA, assuming that the effect of treatment would be revealed by significant "time × treatment" interaction term, where "time" denotes within-individual differences in wild-grown and lab-grown feathers. All reported *P* values are calculated for two-sided tests.

Results

Carotenoid treatment had a significant effect on feather chroma (Table 1; Fig. 1). Lab-grown feathers of carotenoid supplemented birds had significantly (30.6%) higher chroma as compared to the feathers of unsupplemented individuals. Vitamin E supplementation did not affect the change in feather colour.

Discussion

The manipulation of dietary vitamin E availability did not affect the coloration of lab-grown feathers although simultaneous carotenoid supplementation had significant positive effect on the chroma values of these ornaments. These findings do not support the hypothesis, put forward by Hartley and Kennedy (2004), that carotenoid-based ornaments serve mainly as indicators of the abundance of other (uncoloured) antioxidants. Our results also differ from previous findings demonstrating that supplementary feeding with uncoloured antioxidants—melatonin (Bertrand et al. 2006b) and vitamins E and C (Pike et al. 2007) resulted in enhancement of carotenoid-based coloration in captive male zebra finches and sticklebacks, respectively. These discrepancies can be explained either by the different antioxidants, experimental methodology or by different ornaments and species studied.

We acknowledge that also some confounding factors, rather than the absence of biologically meaningful relationships, may be responsible for the lack of effect of vitamin E supplementation on feather coloration. For

instance, it could be argued that the supplemented micellized tocopherols were not at all, or were not easily absorbable for the birds. However, this is unlikely, as it has been shown that micellized vitamin E supplements effectively increase circulating plasma tocopherol levels (Ochoa et al. 1992; Traber et al. 1994).

Another, more likely confounding factor might be that high tocopherol concentration of the base diet masked the effect of vitamin E supplementation. Sunflower seeds are rich in vitamin E (Yoshida et al. 2002), while being a relatively poor source of carotenoids (McGraw et al. 2001). It has been suggested that if α-tocopherol is ingested in large doses, much is not absorbed and is excreted (Halliwell and Gutteridge 2004). Higher doses of vitamin E produce relatively small increases in plasma tocopherol levels in humans (Burton et al. 1998) and barn swallows (*Hirundo rustica*; de Ayala et al. 2006). However, at present it is largely unknown whether and how limiting nutrient vitamin E is in wild birds. Most of the studies reporting the beneficial effects of vitamin E supplementation in birds have concentrated on domestic species under strict artificial diets, or embryos and juveniles, which exhibit extremely high metabolic rates and may experience extensive oxidative stress (e.g. Surai 2002). A single study of wild passerines (de Ayala et al. 2006) found that relatively low physiological doses had small and transient effects on nestling growth and condition in barn swallows, whereas a higher physiological dose did not enhance offspring quality. Plasma α-tocopherol levels of captive greenfinches on sunflower seed diet (20.6–26.8 μg/ml; Hõrak et al. 2004b) are relatively high compared to the concentrations reported in several other wild passerines (< 13 μg/ml; e.g. Biard et al. 2005; de Ayala et al. 2006; Ewen et al. 2006; but see Hõrak et al. 2004a). Thus, we cannot totally exclude the possibility that the effect of vitamin E supplementation in our study had no effect on feather coloration because all birds received already the maximum absorbable concentration of tocopherols from their sunflower seed diet.

We found that while lab-grown feathers were all duller than wild-grown feathers, dietary carotenoid supplementation resulted in increased feather chroma compared to unsupplemented individuals. This result is not surprising and compares favourably with many previous studies (reviewed in Hill and McGraw 2006). The ''bleaching'' of the feather coloration in captivity has been reported previously (discussed in detail e.g. in Hõrak et al. 2004b; Hill and McGraw 2006) and can be most likely ascribed to insufficient carotenoids (or other micronutrients required for carotenoid biotransformations, transportation and deposition to integument) in the diet. The significant effect of dietary carotenoid supplementation has been reported in many different avian species (reviewed in Hill and McGraw 2006). Altogether, the result that increased carotenoid availability is mirrored in sexual ornaments is consistent with the current understanding of the mechanics of carotenoid-based signalling (e.g. Møller et al. 2000; Surai 2002).

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CURRICULUM VITAE

I General

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II Research history

Research interests:
Parasite mediated sexual selection, the cost of immune activation.

Publications:

Hõrak, P., Saks, L., Karu, U., Ots, I., Surai, P.F. & McGraw, K.J. (2004) How coccidian parasites affect health and appearance of greenfinches. *Journal of Animal Ecology* 73: 935–947.

Hõrak, P., Saks, L., Karu, U. & Ots, I. (2006) Host resistance and parasite virulence in greenfinch coccidiosis. *Journal of Evolutionary Biology* 19: 277–288.

Saks L., Karu U., Ots, I., Hõrak, P. (2006) Do standard measures of immunocompetence reflect parasite resistance? The case of Greenfinch coccidiosis. *Functional Ecology* 20: 75–82.

Hõrak, P., Zilmer, M., Saks, L., Ots, I., Karu, U. & Zilmer, K. (2006) Antioxidant protection, carotenoids, and the costs of immune challenge in greenfinches. *Journal of Experimental Biology* 209: 4329–4338.

Hõrak, P., Saks, L, Zilmer, M., Karu, U. & Zilmer, K. (2007) Do dietary antioxidants alleviate the cost of immune activation? An experiment with greenfinches. *American Naturalist* 170: 625–635.

Karu, U., Saks, L., Hõrak, P. (2008) Plumage coloration is not affected by vitamin E supplementation in male greenfinches. *Ecological Research,* in press.

Conference theses:

Hõrak, P., Saks, Karu, U., Ots, I., Surai, P.F. and McGraw, K.J. "How coccidian parasites affect health and appearance of greenfinches (*Carduelis Chloris*)". 9th Congress of the European Society for Evolutionary Biology. 18–24 August 2003 Leeds, UK. (poster)

Saks, L., Karu, U., Ots, I., Hõrak, P. "Parasite resistance and immunocompetence in greenfinch-coccidiosis model". 10th Congress of the International Society for Behavioral Ecology. Symposia of parasites and immune function. 10–15 July 2004 Jyväskylä, Finland. (poster)

Hõrak, P., Karu, U., Saks, L., Ots, I. "Does the virulence of coccidian infection in greenfinches depend on hosts, parasites, or their interaction?" 10th Congress of the International Society for Behavioral Ecology. 10–15 July 2004 Jyväskylä, Finland. (poster)

Hõrak, P., Saks, L., Ots, I., Karu, U. "Immune function, carotenoids, and antioxidant defences in greenfinches". 24th International Ornithological Congress, 13–19 July 2006 Hamburg, Germany. (poster)

Hõrak, P., Saks, L., Zilmer, M., Karu, U. and Zilmer, K. "Immune activation increases and carotenoids reduce oxidative damage in greenfinches". 11th Congress of the European Society for Evolutionary Biology. 20–25 August 2007. Uppsala, Sweden. (poster)

Karu, U., Saks, L., Hõrak, P. "Plumage coloration is not affected by vitamin E supplementation in male greenfinches". 11th Congress of the European Society for Evolutionary Biology. 20–25 August 2007. Uppsala, Sweden. (poster)

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Peamised uurimisvaldkonnad:
Parasiitide vahendatud suguline valik, immuunaktivatsiooni hind.

Publikatsioonide loetelu:

Hõrak, P., Saks, L., Karu, U., Ots, I., Surai, P.F. & McGraw, K.J. (2004) How coccidian parasites affect health and appearance of greenfinches. *Journal of Animal Ecology* 73: 935–947.

Hõrak, P., Saks, L., Karu, U. & Ots, I. (2006) Host resistance and parasite virulence in greenfinch coccidiosis. *Journal of Evolutionary Biology* 19: 277–288.

Saks L., Karu U., Ots, I., Hõrak, P. (2006) Do standard measures of immunocompetence reflect parasite resistance? The case of Greenfinch coccidiosis. *Functional Ecology* 20: 75–82.

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Konverentside teesid:

Hõrak, P., Saks, Karu, U., Ots, I., Surai, P.F. and McGraw, K.J. "How coccidian parasites affect health and appearance of greenfinches (*Carduelis Chloris*)". 9th Congress of the European Society for Evolutionary Biology. 18–24 august 2003 Leeds, Suurbritannia. (poster)

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Hõrak, P., Saks, L., Ots, I., Karu, U. "Immune function, carotenoids, and antioxidant defences in greenfinches". 24th International Ornithological Congress, 13–19 juuli 2006 Hamburg, Saksamaa. (poster)

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