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**TUUL SEPP**

Hematological health  
state indices of greenfinches: sources  
of individual variation and responses  
to immune system manipulation



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## LIST OF ORIGINAL PAPERS

1. Sarv, T.; Hõrak, P. (2009). Phytohaemagglutinin injection has a long-lasting effect on immune cells. *Journal of Avian Biology*, 40, 569–571.
2. Sepp, T., Sild, E., Blount, J., Männiste, M., Karu, U., Hõrak, P. (2012) Individual consistency and covariation of measures of oxidative status in avian blood. *Physiological and Biochemical Zoology*, 85, 299–307.
3. Sepp, T.; Sild, E.; Hõrak, P. (2010). Hematological condition indexes in greenfinches: effects of captivity and diurnal variation. *Physiological and Biochemical Zoology*, 83, 276–282.
4. Sepp, T.; Karu, U.; Sild, E.; Männiste, M.; Hõrak, P. (2011). Effects of carotenoids, immune activation and immune suppression on the intensity of chronic coccidiosis in greenfinches. *Experimental Parasitology*, 127, 651–657.
5. Sepp, T., Karu, U., Blount, J., Sild, E., Männiste, M., Hõrak, P. (2012). Coccidian infection causes oxidative damage in greenfinches. *PLoS ONE*, 7 (5): e36495.
6. Hõrak, P.; Sild, E.; Soomets, U.; Sepp, T.; Kilk, K. (2010). Oxidative stress and information content of black and yellow plumage coloration: an experiment with greenfinches. *Journal of Experimental Biology*, 213, 2225–2233.

### Author's contribution to the papers

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

# I. INTRODUCTION

## I.1. Immunoecology – a multidisciplinary approach

All organisms are infected by numerous parasite species, many of which are host-specific (Schmid-Hempel, 2011). Hosts vary considerably in their susceptibility to infection. Finding the causes of this variation has become one of the key questions to both ecologists and evolutionary biologists, since the role of parasites and pathogens in the evolution of life-history- and signal traits is drawing more and more attention. Immune system protects the organisms against this vast array of intruders. To detect and destroy parasites, immune system constructs sophisticated recognition and memory pathways, releases harmful substances and increases metabolism. One common factor that links all classes of host defenses is that they require resources that the host might otherwise have used for some other function (Sheldon & Verhulst, 1996). These resources might be directly needed for mounting an immune response, but also for damage control after the immune response has taken place. Immune defenses thus have their costs – maintaining an effective immune system and conducting an immune response might come at the expense of some other function vital for the life-history of the organism (Zuk & Stoehr, 2002). This approach is the basis of the expanding new discipline – immunoecology (Lee, 2006; Sheldon & Verhulst, 1996).

The primary goal of immunoecology is to find out the factors, both extrinsic and intrinsic, leading to changes in immune system function, and understand how these changes affect disease susceptibility (Demas & Nelson, 2012). For example, it has been shown that the magnitude of immune response to microbial challenges might depend on seasonality (Lozano & Lank, 2003), temperature (Adamo & Lovett, 2011) and population density (Müller *et al.*, 2006), but also on intrinsic factors such as age (Belloni *et al.*, 2010), sex (Fargallo *et al.*, 2007) or stress level (Martin, 2009). Immune responses are deeply integrated to host physiology, at the same time being sensitive to environmental variation and can therefore be important indicators of overall physical condition (O'Neal & Ketterson, 2012). A multidisciplinary approach to immune system research gives valuable knowledge about the functioning of an organism in addition to narrowly specialized immunological studies.

The aim of immunoecological research is to discover the mechanisms that link immune function to other vital functions of an organism, such as sexual signalling, reproduction, growth or maintenance. Eventually, such an approach helps to explain the causes and consequences of variation of individual phenotypic quality in natural populations.

## 1.2. Costs of immune response

Viewing the immune system in the context of the whole organism and its life-history, we can start to understand why hosts remain susceptible to parasites and why immune responses vary. Although it has now been accepted that immune responses are costly (for examples, see Moreno-Rueda, 2011; Moret & Schmid-Hempel, 2000; Sorci & Faivre, 2009), the question about the currencies used for paying this cost has remained poorly understood. One reason for this gap in our knowledge might be the complicated nature of immune system, which makes assessing the magnitude of immune response and accompanying costs difficult.

Immune system consists of innate and adaptive arms that are tightly integrated with each other and fulfill four main tasks: immunological recognition, immune effector functions, immune regulation and immunological memory (Murphy *et al.*, 2008). Cross-regulatory mechanisms between different parts of immune system have been described in several levels of immunity (Boughton *et al.*, 2011; Demas *et al.*, 2011), giving more reasons to believe that maximal immune response is not always optimal. For example, activation of one component of immunity might lead to suppression of another component (Graham *et al.*, 2005). This also means that single measures of immunity are rarely sufficient to capture the complexity of immune response or individual's health state (Demas & Nelson, 2012). In accordance with this, recent works have not concentrated on single measures of immunity, but measured constitutive and induced immune responses from both innate and adaptive sides of immunity (see O'Neal & Ketterson (2012) for review).

Although the traditional view of animal ecologists has been that the costs involved in immune response are mainly energetic (Lochmiller & Deerenberg, 2000; Sheldon & Verhulst, 1996), an alternative hypothesis, proposing that costs of immune responses are primarily caused by the accompanying immunopathological tissue damage, is gathering popularity (Dowling & Simmons, 2009; Sorci & Faivre, 2009). The innate immune system protects the organism by producing reactive oxygen species in a process called oxidative burst (Murphy *et al.*, 2008). These oxygen species are highly reactive and destroy pathogens by damaging their biomolecules. Reactive species are not pathogen-specific and can also damage host tissues if there are not enough protective antioxidants present (Halliwell & Gutteridge, 2007). Oxidative stress is a situation when the balance of pro-oxidants and antioxidants is shifted towards pro-oxidants, causing oxidative damage to organisms' tissues (Sies, 1997). Oxidative stress is believed to play an important role in senescence, expression of signal traits and sperm performance, hence affecting reproduction and survival (Buttemer *et al.*, 2010; Cohen *et al.*, 2010; Costantini *et al.*, 2010; Hall *et al.*, 2010; Losdat *et al.*, 2011). Therefore, oxidative stress may be one of the mechanisms that link immune function with life-history traits.



### 1.3. Oxidative stress and ecological research of animals

To study the role of oxidative stress as a mediator of life-history trade-offs, the oxidative status of an organism has to be reliably measured and properly interpreted. Similarly to immunoeological research, several approaches and proxies have been used. It is a quite common approach to measure the levels of different antioxidants (Almbro *et al.*, 2011) or overall antioxidant potential of a tissue (Yeum *et al.*, 2004). However, the problem may arise with the interpretation of results (Costantini & Verhulst, 2009). For example, high levels of plasma antioxidant capacity (TAC) is sometimes interpreted as beneficial redox state (Tummeleht *et al.*, 2006), although it could reflect adaptive and compensatory responses to oxidative stress rather than optimal health condition (Hörak & Cohen, 2010; Prior & Cao, 1999). As an alternative to antioxidant levels, oxidative status can be assessed on the basis of overall oxidative potency of a given specimen (e.g. blood plasma) (Erel, 2005) or on the concentration of individual oxidants (for example Wood *et al.*, 2009). Perhaps the most ecologically relevant approach would be to measure oxidative damage (Halliwell & Whiteman, 2004), since it would reflect the situation when antioxidants have been unable to defend the tissues from oxidants. Depending on studied system, all of these methods have their advantages and disadvantages, but proper understanding of the mechanisms and interpretation possibilities of individual methods is crucial. Finding a combination of biomarkers of oxidative status that can be applied to typical research models is one of the most actual problems of oxidative stress and immune system research.

The main insight of animal ecologists to the biology of oxidative stress comes from avian studies (Costantini, 2008). The increasing interest of avian ecologists in oxidative stress arises partly from the intriguing hypothesis that oxidative status of birds may shape the development of their sexual ornamentation (Lozano, 1994; von Schantz *et al.*, 1999). Most conspicuous avian colors are produced by carotenoids. Although animals cannot synthesize carotenoids *de novo*, these pigments are extraordinarily common components of the color signals used in sexual communication, signaling between offspring and their parents, and in warning colors (Hill & McGraw, 2006; Møller *et al.*, 2000). Carotenoids are not only used in ornamental coloration, but also for several other functions, for example defense from solar radiation and immunomodulation (McGraw *et al.*, 2011). It is also suggested that carotenoids may act as important antioxidants in organisms (Møller *et al.*, 2000; von Schantz *et al.*, 1999). However, the role of carotenoids as antioxidants and immunomodulators has recently been debated (Costantini & Møller, 2008; Hartley & Kennedy, 2004; Pérez-Rodríguez, 2009), partly because the mechanisms by which carotenoids modulate oxidative balance or immunity are poorly known.

Although carotenoids are perhaps the most intensively studied pigments in birds' plumage, the most common pigments are melanins (McGraw, 2006).

Many birds develop melanin-based feather patches that signal mate quality or social dominance (Andersson, 1994; Jawor & Breitwisch, 2003; McGraw, 2003; McGraw, 2006). Unlike carotenoids that are directly obtained from food, melanin pigments in animals are synthesized in specialized cells, called melanocytes. Because of the different processes associated with melanin and carotenoid color production, different factors might be responsible for mediating the honesty of melanin-based signal traits compared with carotenoid colors (McGraw, 2008). An idea that similarly to carotenoids, melanin pigments can also potentially function as antioxidants has lately reached the ecological literature (Griffith *et al.*, 2006; Moreno & Møller, 2006).

There are also other pathways that may possibly link melanin-based signals to oxidative status of a bird. First, melanogenesis is an oxidative process that may act as a potential source of reactive oxygen species (Smit *et al.*, 2008). Second, an interesting new hypothesis suggests that since the key intracellular antioxidant– glutathione (GSH) – inhibits eumelanogenesis, eumelanin-based black ornaments may signal the bearer’s ability to cope with oxidative stress (Galván & Alonso-Alvarez, 2008). According to the honest advertisement hypothesis (Zahavi, 1975), only those individuals who manage to keep their oxidative stress level under control without high levels of GSH are able to produce eumelanin-based ornaments. However, the links between oxidative damage, systemic GSH levels, and melanin- and carotenoid-based feather coloration are not yet clearly established.

#### **I.4. Aims of the thesis**

The main aims of current thesis were (1) to assess the suitability and utility of several widely used methods in ecological research of immunity and oxidative balance systems, and (2) to study some of the most intriguing questions in avian immunoeology. In the first part, I tested the long-term impact of a classic immunoeological technique (PHA skin test), correlations between several indexes of oxidative status and their repeatability in time, and the suitability of wild-caught captive greenfinches for ecophysiological research. In the second part, I studied the association between carotenoids and immune function, oxidative costs of mounting an immune response, and the information content of plumage coloration.

In the first study, I tested the effect of a common immunostimulant on leukocyte profiles of greenfinches. Leukocyte profiles are easily measurable, yet informative health state indices, reflecting the current investment in immune defense (Dein *et al.*, 1986) or stress level (Davis *et al.*, 2008). To study the trade-offs between immune function and other vital functions, ecologists need simple and reliable methods that make it possible to assess the immune function of an organism. Because of its ease of use in the field, the most widely used immunological method in avian studies is the phytohaemagglutinin (PHA) skin

test (Martin *et al.*, 2006). PHA is a plant lectin which functions in plants as an antimicrobial and anti-herbivore toxin. Subcutaneous injection of PHA to animals produces a localized swelling response involving local infiltration of tissue by most types of immune cells. The magnitude of this swelling is widely used as an indicator of immunocompetence or general health state (Tella *et al.*, 2008), although it has been argued that more swelling might not always show better health (Martin *et al.*, 2006). Although this method has been widely used for several decades, the mechanisms involved in producing the response to PHA and its long-term impact to organism's physiology are poorly known. Accordingly, I tested the long-lasting physiological impact of PHA on leukocyte profiles of greenfinches (**Paper I**).

In the next study, I continued my methodological investigation with several widely used indices of oxidative status. Reactive oxygen species production is a consequence of cellular metabolism and immune responses. Therefore, oxidative stress might be a proximate mechanism responsible for the emergence of trade-offs related to the evolution of life-history (Monaghan *et al.*, 2009). To answer ecological questions about the role of oxidative stress, it is crucial to find a combination of biomarkers of oxidative status that can be applied to typical wild animal models such as small birds. These biomarkers should be tested for confounding and modifying factors, and for the individual consistency in time (Hörak & Cohen, 2010). Because of specific restrictions of many ecological research models, such as the availability of only small amounts of blood and the requirement of nonlethality, it is also important to be aware of covariation between biomarkers in order to avoid measurement of redundant parameters (Costantini *et al.*, 2011). The aim of my second study was to describe covariation, individual consistency and sensitivity to changes in oxidative stress level of eight widely used parameters of oxidative status (**Paper II**).

The majority of immunoeological research, especially development and adjustment of novel methods, is carried out under laboratory conditions. In addition to individual consistency of studied parameters, it is also important to know how different parameters are affected by captivity, if one wants to generalize the information obtained from studying captive animals to natural situations. The few previous studies about the effect of captivity on the physiology of birds have yielded contradictory results. In some occasions, confinement of wild birds to captivity has considerably increased their stress levels (Ruiz *et al.*, 2002), at the same time, other species show stress levels comparable with or even lower than in the nature (Buehler *et al.*, 2008; Ewenson *et al.*, 2001; Piersma *et al.*, 2000). It is possible that some bird species tolerate captivity better than others; therefore, it is important to test the effects of captivity on classical model species. Accordingly, in the third study, I compared various hematological condition parameters between wintering wild greenfinches and greenfinches that had been kept in indoor aviaries for different periods of time, analyzing also the diurnal variation of studied parameters (**Paper III**).

To evaluate the costs and individual variation of immune responses, it is important to find model systems that reflect the situation in the nature and are easy to manipulate under experimental conditions. One of such systems is the coccidiosis infection in greenfinches. Coccidians are directly transmitted protozoans that cause massive production loss in poultry industry (Zhu *et al.*, 2000) and affect fitness in wild birds (reviewed by Hōrak *et al.*, 2004; Pap *et al.*, 2011). All greenfinches that have been examined in our laboratory have been naturally infected with this parasite and the infection levels are easily traceable. In the **Paper IV**, I used the greenfinch coccidiosis model to assess the effect of three different manipulations on infection levels. First, I investigated how dietary carotenoid supplementation affects the dynamics of natural infection. The association between carotenoids and immune function is one of the most intriguing questions in immunoeecology. The link between carotenoid-based ornamentation and immune function was first suggested by Lozano (1994) and later developed by von Schantz *et al.* (1999). Experimental studies assessing the role of carotenoids in immune response have produced contradicting results. Assessing the ability of carotenoids to affect real infections (compared with responses to artificial antigens or non-specific immune parameters) might help to understand the potential health benefits of these pigments. Other manipulations – immune suppression and immune activation – were performed in order to amplify the potential effects of carotenoid supplementation. I tested whether possible immunostimulatory effects of carotenoids would emerge when immune system is suppressed by a synthetic corticosteroid, dexamethasone (DEX), and whether priming of immune system with phytohaemagglutinin (PHA) would induce stronger immune response against coccidiosis.

Continuing with greenfinch coccidiosis, I tested if infection with this parasite causes oxidative damage (**Paper V**). One of the main concepts in immunoeecology is that individual variation in immune responsiveness is caused by the costs of immune responses to the hosts. These costs might involve oxidative damage resulting from the excessive production of reactive oxygen species during immune response. To assess the potential oxidative damage accompanying coccidian infection, I used plasma malondialdehyde (MDA) levels. MDA is an end-product of peroxidative decomposition of unsaturated lipids; it is also mutagenic and cytotoxic and can damage membrane proteins (Halliwell & Gutteridge, 2007). The level of MDA is often considered as a presumptive marker of oxidative stress. In addition to assessing the effects of experimental treatment and reinfection to MDA levels, I also tested whether higher individual plasma levels of MDA are associated with better resistance to infection, because birds use oxidative destruction of parasites to control infection level.

In the final case study (**Paper VI**) I added another important pigment – melanin – to my scope, asking what kind of information carotenoid- and melanin-based plumage ornaments might convey. There has been much interest and controversy over the factors that keep carotenoid- and melanin-based

ornaments reliable as signals of individual quality (Griffith *et al.*, 2006; McGraw, 2008). Both types of pigments are claimed to be sensitive to oxidative stress (Griffith *et al.*, 2006; Moreno & Møller, 2006). To test the new hypothesis about the role of an important intracellular antioxidant, glutathione (GSH) in the development of melanin-based ornaments (Galván & Alonso-Alvarez, 2008), I manipulated dietary carotenoids and systemic GSH levels, and recorded the effects of these treatments upon coloration of feathers grown during experiment. To clarify how these treatments affect oxidative status of birds, I monitored changes in different health state indices, including a measure of oxidative damage, MDA, during the experiment.

## 2. MODEL SYSTEM

In immunoeological research, trade-offs are not reserved only to organisms' physiology. The necessity to give up something in order to gain in other areas comes also up in designing the studies. If we want to extrapolate the results obtained in the laboratory to the real world, the experimental conditions should be kept as close to natural situation as possible. At the same time, to establish causal links, the model systems should be kept as simple as possible. This is complicated in natural environments, where numerous factors are simultaneously affecting the studied variables. If we gain on generalization potential, we may lose in reliability of interpretation and vice versa. As a compromise, I have used wild species and natural infections in my studies, but conducted the experiments in laboratory, which allows keeping the environmental conditions homogenous and enables constant monitoring. Therefore, my model system composes of wild-caught captive greenfinches that are naturally infected with coccidian parasites.

Greenfinches are medium-sized (ca 28 g), sexually dichromatic gregarious seed-eating passerines native to western Palearctic region. Their melanin- and carotenoid-based plumage makes them good model species for studying the honesty of signal traits. Males are larger and more colorful than females, with older males developing olive-green plumage on the back, bright or greenish yellow colour on the breast, and striking yellow markings on the primaries, primary coverts and sides of the tail feathers (Cramp & Perrins, 1994). 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). Carotenoid-based plumage coloration of male greenfinches is sexually selected (Eley, 1991) and sensitive to infections (Lindström & Lundström, 2000; Merilä *et al.*, 1999). Greenfinches tolerate captivity easily and have often been used as a model of passerine species in ecophysiological research (for example Aguilera & Amat, 2007; Lilliendahl, 2000; Lindström *et al.*, 2001; Peters *et al.*, 2008; Sild *et al.*, 2011).

In birds, intestinal coccidiosis is one of the most easily traceable natural infections. Coccidians are directly transmitted protozoans of the genus *Isospora* that cause massive production loss in poultry industry (e.g., Allen & Fetterer, 2002a; Zhu *et al.*, 2000) and affect fitness in wild birds (reviewed by Hōrak *et al.*, 2004; Pap *et al.*, 2011). These obligate intracellular parasites inhabit the intestinal epithelium and inhibit the uptake of essential dietary components, including fat-soluble antioxidants such as vitamin E and carotenoids (Allen & Fetterer, 2002b; Hōrak *et al.*, 2004). All of the greenfinches that have been examined in our laboratory have been naturally chronically infected with coccidians, therefore we can assume that some kind of tolerance has evolved. At the same time, the pathogenicity (which can ultimately lead to host's death) of *Isosporan* coccidiosis is well documented (Box, 1977; Giacomo *et al.*, 1997; Sironi, 1994) and it is likely that these parasites have been an important

evolutionary force for greenfinches. Coccidiosis of wild birds is becoming an increasingly popular model of parasite-mediated selection, particularly in the context of carotenoid-based ornaments and oxidative stress ecology (for example Baeta *et al.*, 2008; Hõrak *et al.*, 2004; Pap *et al.*, 2009; Pap *et al.*, 2011).

## 3. RESULTS AND DISCUSSION

### 3.1. Testing the long-term impact of PHA on leukocyte profiles

In the first paper I tested the long-term impact of a phytohaemagglutinin on leukocyte profiles of captive greenfinches. PHA skin test is a classic immunological technique: subcutaneous injection of PHA induces localized swelling response and the magnitude of this response is widely used as an indicator of immunocompetence (Tella *et al.*, 2008) and inflammation (Martin *et al.*, 2006). Since this plant lectin activates immune system, it has also been used in studies trying to assess the physiological costs of mounting an immune response (Costantini & Dell'Omo, 2006; Martin *et al.*, 2003; Perez-Rodriguez *et al.*, 2008). These and several other studies have shown that activation of immune system in response to PHA injection is costly to the organism, although in relatively short time scale (24 to 72 hours). However, there is some reason to expect long-term impact of PHA on animal physiology (Hörak *et al.*, 2000). Here I measured the change in greenfinch leukocyte profiles up to 30 days after PHA injection.

To assess the health state of the birds, four leukocytic parameters were assessed: total white blood cell count (WBC), heterophil concentration, lymphocyte concentration, and heterophil/lymphocyte ratio (H/L). Leukocytosis and heterophilia can be associated with localized or systemic infections caused by a wide spectrum of infectious agents and noninfectious etiologies (for example injuries, toxicities, stress) (Campbell & Ellis, 2007). H/L ratio is considered a reliable indicator of stress level in birds (Davis *et al.*, 2008).

Six captive greenfinches were injected in the wing web with PHA solution, at the same time control group (7 birds) received saline solution injection. For counting leukocytes, blood smears were prepared on the day of injections, three days after injections, and thirty days after injections. Three days after injections, heterophil concentrations and H/L ratio increased both in control and experimental group, but more among PHA-injected birds. The impact of PHA was even more pronounced thirty days after injections, when heterophil counts of PHA injected birds were still 29% higher than those of saline-injected controls. At the same time the difference between PHA- and saline-injected birds in H/L ratios was 37%. Post-injection values of total leukocyte count or lymphocyte concentration did not differ between treatment groups, so the elevated H/L level arises mainly from the increase of heterophil concentration.

Why does PHA cause prolonged heterophilia? Several different physiological explanations are possible. The most likely cause would be an inflammatory response, mediated by pro-inflammatory cytokines. These cytokines mediate changes in glucocorticoids, acute-phase proteins, and recruit monocytes and heterophils from the bone marrow (Leshchinsky & Klasing, 2001). Elevated heterophil levels may be harmful for the organism, damaging host



tissues in the process of oxidative burst and degranulation (Harmon, 1998). Although I cannot confirm that the observed change in leukocytic parameters implied serious long term health impact, it would be important to consider the possible long-term impact of PHA injections to immune system when PHA skin test is used in studies monitoring changes in individual physiological condition.

### **3.2. Investigating the measures of oxidative status**

The aim of my second study was to describe covariation and individual consistency of different parameters of oxidative status in greenfinches. A prevailing paradigm in biomedical research is that the oxidative status of an individual can be evaluated based on the composition of its body fluids and tissues. Implementing these approaches to ecological research has generated considerable confusion, mainly because of the difficulties of interpretation (Isaksson *et al.*, 2011), and so far, there is no agreement on what parameters should be measured in typical model species of animal ecology and how the obtained results should be interpreted.

I included 56 greenfinches and eight widely used indexes of oxidative status in my study. These include two markers of plasma antioxidant potential – total antioxidant capacity (TAC) and oxygen radical absorbance (OXY) – and concentrations of one lipophilic (carotenoids) and two hydrophilic (uric acid and ascorbate) antioxidants in plasma. From the erythrocytes, I measured concentration of total glutathione (GSH) – a thiol tripeptide, which is considered a key intracellular antioxidant – and superoxide dismutase (SOD; an enzymatic antioxidant) activity. For the assessment of oxidative damage, plasma malondialdehyde (MDA) levels were used, measured by high-performance liquid chromatography (HPLC). MDA is an end product of peroxidative decomposition of unsaturated lipids; it is also mutagenic and cytotoxic, can damage membrane proteins (Halliwell & Gutteridge, 2007), and is often considered a presumptive marker of OS (e.g., Mateos *et al.*, 2005). I asked whether any of the measured parameters correlate with each other and are individually consistent in time (measured over 8- and 16-day period). Additionally, I controlled if the measured parameters would be sensitive to manipulation of oxidative status of birds with a synthetic mimetic of the antioxidants superoxide dismutase (SOD) and catalase. This manipulation was expected to indicate which of the studied biomarkers are most sensitive to experimental reduction of systemic ROS levels.

Three of the six parameters of oxidative status – TAC and carotenoids in plasma and GSH in erythrocytes – were individually consistent over the 8-d period, while none of the variables were significantly correlated over 16 d. Therefore, studies considering the long-term aspects of wellbeing or components of fitness should prefer measurement of individually more stable traits like carotenoids, TAC, and GSH to measurement of MDA, OXY, and uric acid.

However, caution is required in extrapolating these findings to other species (for example, see Saino *et al.*, 2011). Because our sample sizes enabled us to detect only medium and high effect sizes for most of the traits, we cannot exclude the possibility that smaller but still biologically important long-term repeatabilities of studied traits exist.

None of the parameters of oxidative status correlated significantly with others. This suggests that all of these biomarkers contain potentially unique information and none of the studied parameters should be considered redundant when composing a battery of markers for the assessment of oxidative status of individuals. Such correlations can again depend on the taxon, ecology, and diet of the studied species (Cohen *et al.*, 2009).

Treatment with a mimetic of SOD and catalase did not affect any parameters of oxidative status or body mass. It is possible that our birds appeared in a good oxidative balance and superior health condition, so that any administration of extra antioxidants would have been redundant. This study leaves open the question of whether the lack of significant associations between the studied variables might have resulted from the generally mild experimental conditions, characterized by lack of oxidative challenges. This reinforces the idea that further studies of the parameters of oxidative status in wild animals would benefit from the experimental induction of oxidative stress.

### **3.3. How are different health state indexes affected by captivity?**

The greenfinch is becoming an increasingly popular model species in eco-physiological research not only because of its carotenoid-based plumage components and relatively large size, but also because of its good captivity tolerance. The question about whether and how captivity affects different physiological parameters of greenfinch is therefore of major importance for interpreting the results of such studies. In this study, I compared various hematological condition parameters and peripheral leukocyte concentrations between wintering wild greenfinches and greenfinches that had been kept in indoor aviaries. Additionally, I assessed the diurnal variation of these parameters in order to clarify the importance of this potentially confounding factor.

Blood from captive birds was collected on 32th and 58th day of captivity. On the second occasion, half of the captive birds were bled in the evening, other subset was bled the next morning to assess the diurnal variation in studied parameters. Free-living birds were caught and immediately sampled on the equivalent of 31th day in captivity for captive birds. In addition to leukocytic parameters (total white blood cell count (WBC), heterophil and lymphocyte concentration and heterophil/lymphocyte ratio (H/L)), I also measured body mass, plasma triglyceride, and total protein levels as indicators of nutritional state (Hörak *et al.*, 1999), and hematocrit as a marker of hydration, work load,

and general health state (Campbell & Dein, 1984; Fair *et al.*, 2007). Considering the increasing interest of animal ecologists to issues related to oxidative stress, I added plasma total antioxidant capacity (TAC) and concentrations of two antioxidants, carotenoids and uric acid, to the assessed parameters.

The most prominent differences between wild and captive greenfinches emerged in plasma uric acid and carotenoid concentrations, which were consistently higher among wild birds. Lower carotenoid levels of captive birds can be ascribed to the low carotenoid content of the diet in captivity, that consists of sunflower seeds (McGraw *et al.*, 2001). This problem can be easily overcome with dietary carotenoid supplementation, which enables the adjustment of the plasma carotenoid concentrations of captive birds to desirable levels (e.g., Aguilera & Amat, 2007). High uric acid levels of wild greenfinches may show that as a result of their unbalanced diet captive birds had to rely on different mechanisms for maintaining their redox balance, depleting their plasma uric acid to a greater extent. Notably, I did not detect any diurnal variation in uric acid or TAC values. This indicates that these parameters seem to be convenient for assessment of variation of physiological condition in birds as a result of a lack of confounding interference of capture time.

Indexes of nutritional state (body mass, plasma triglycerides, and total protein) did not reveal any systematic differences between wild and captive birds. Instead, remarkable diurnal variation was expressed under captive conditions, with higher parameter values in birds sampled in the evening. Such patterns are expected, given that plasma triglyceride levels indicate the amount of lipids absorbed during the few hours before blood sampling (Jenni-Eiermann & Jenni, 1998) and that body mass depends on the amount of food in the alimentary tract.

Hematocrit of wild greenfinches was higher than that of captive birds sampled on day 32 of captivity. After 2 months in captivity, the hematocrit values had further increased, being higher in the morning than in the previous evening. One might assume that wild birds must work harder to find food and maintain body temperature in winter than captive birds. Increase of hematocrit toward the end of the captivity period might relate to increasing day length (13 h at the end of March vs. 10.5 h at the end of February), which leaves more time for physical activity and feeding. Absolute and differential leukocyte concentrations did not reveal any consistent pattern with respect to sampling event. Thus, the data on leukocyte differentials suggest that the confinement of greenfinches to captivity in this study did not cause a detectable increase in stress levels, at least after 1 or 2 months spent in the aviary. These findings, along with those about most of the blood parameters measured in this study, are encouraging with respect to the validity of the physiological data collected in captive birds.

### 3.4. Carotenoids modulate immune response to chronic infection

Links between immune function and carotenoids have been experimentally demonstrated in several bird species, either by the positive effect of carotenoid supplementation or by stimulation of immune system that depleted carotenoid stores (for example Aguilera & Amat, 2007; Fitze *et al.*, 2007; McGraw *et al.*, 2006; Perez-Rodriguez *et al.*, 2008; Stirnemann *et al.*, 2009). Most of these studies have used artificial antigens, which makes interpretation of the results complicated – maximum immune responses are not necessarily optimal or best for an organism as a whole (e.g. Viney *et al.*, 2005) and enhancement of one arm of the immune system may down-regulate another arm (Graham *et al.*, 2005). In this study I assessed the effect of carotenoids on the immune system on functional level, observing the changes in chronic coccidiosis infection intensity in response to three different manipulations – carotenoid supplementation, immune activation and immune suppression. Examining the role of carotenoids in immune response through controlled immune suppression and immune activation experiments has remained so far unexplored. For instance, it may appear that the impact of carotenoids on natural infection levels is detectable only during immune suppression caused by stress or after previous activation of immune system.

93 male greenfinches naturally infected with coccidian parasites were included in the experiment. Carotenoids were administered in three different doses (0 µg/ml for control group, 8 µg/ml and 16 µg/ml for experimental groups), because the physiological and immunomodulatory effects of carotenoids can be dose-dependent (Alonso-Alvarez *et al.*, 2004; Krinsky & Johnson, 2005). Immune system was suppressed with a synthetic corticosteroid, dexamethasone (DEX). In birds, DEX-induced immune suppression has been previously used for studying susceptibility to infections (Huff *et al.*, 1999), validation of immunotoxicological techniques (Smits & Williams, 1999), and manipulation of corticosterone levels (e.g., Rich & Romero, 2005), however, to my knowledge, DEX has been never used to study the immunomodulatory effects of carotenoids. Immune system was activated with phytohaemagglutinin (PHA), a plant lectin that activates the same cytokines that are involved in immune response against coccidiosis (Lillehoj, 1998). To monitor the effects of manipulations, several health state indices were assessed: changes in infection intensity, leukocytic parameters (total white blood cell count (WBC), heterophil and lymphocyte concentration and heterophil/lymphocyte ratio (H/L)), body mass, and swelling response to PHA.

All experimental treatments had clear physiological effects. Dietary carotenoid supplementation increased plasma carotenoid levels. This effect was, however, independent of dietary carotenoid concentration, which may explain the lack of dose-dependent effect of carotenoids on other studied parameters. DEX suppressed T-cell mediated swelling response to PHA and also reduced

body mass and increased peripheral heterophil concentrations. The higher heterophil numbers of the DEX-injected birds can be considered as a generic expression of a stress response, and should not indicate stronger immunity, because DEX has been predicted to cause heterophilia through releasing the heterophils which are normally sequestered against the blood vessel walls into general circulation, while simultaneously blocking the maturation and release of T-cells from their primary and secondary tissues of origin (Smits & Williams, 1999). Immune activation with PHA caused a swelling response and also increased heterophil count. The effect of immune stimulation with PHA on body mass depended on the dietary carotenoids: among un-supplemented birds PHA decreased mass while among the carotenoid-supplemented birds, PHA had no effect on mass. This finding suggests that carotenoid supplementation can extenuate the physiological impact of immune activation.

The main aim of this study was to test for the involvement of carotenoids in modulation of immune response. Carotenoid-supplemented birds did not develop stronger swelling response to PHA than un-supplemented birds, neither did PHA injection deplete plasma carotenoid levels. This result is similar to previous experiments on greenfinches in our laboratory but contradicts five other avian studies using different immunostimulants (reviewed by Perez-Rodriguez *et al.*, 2008). It is possible that the timing of measurement is important in detection of the carotenoid depletion by immune challenge. Treatments with DEX, PHA or carotenoids had no significant main effects on the development of coccidiosis. It seems that the DEX treatment confounded the effects of carotenoids and PHA. This implies that the course of immune response to chronic infections is likely affected by glucocorticoids and the immunosuppressive effects of stress might confound the results of the studies trying to assess the role of carotenoids in immune response. When birds treated with DEX were excluded, it appeared that immune stimulation by PHA suppressed the infection, but only among carotenoid-supplemented birds. Suppression of coccidiosis by PHA may be ascribed to the priming of the immune system by this immunostimulatory protein. The effects of PHA on coccidiosis emerged only among carotenoid-supplemented birds, which supports the concept of anti-parasitic effect of carotenoids (reviewed for example in McGraw *et al.*, 2006).

### **3.5. Coccidian infection causes oxidative damage**

A recent hypothesis proposes that the costs of immune responses are primarily caused by the accompanying immunopathological tissue damages (Dowling & Simmons, 2009; Sorci & Faivre, 2009; von Schantz *et al.*, 1999), but the tests of this hypothesis in captive and wild birds have yielded contrasting results (reviewed by Costantini & Møller, 2009). The aim of this study was to test whether experimental infection with coccidians generates oxidative damage in

greenfinches. Plasma malondialdehyde (MDA) levels were used for the assessment of potential oxidative damage. MDA levels were measured by high-performance liquid chromatography, which is considered a more reliable method than the widely used colorimetric TBARS assay (Halliwell & Gutteridge, 2007). Additionally I asked whether higher individual plasma levels of MDA are associated with better resistance to infection. This hypothesis is based on oxidative destruction of Eimerian coccidians in poultry (Allen & Fetterer, 2002a). In addition to changes in infection intensity, I measured plasma antioxidant potential by two corresponding markers (TAC and OXY), body mass, plasma triglycerides and carotenoids in order to monitor antioxidant defences and general physiological and nutritional impact of infection.

All of the birds were infected with isosporan coccidians at capture. At the beginning of the experiment, all birds were treated with an anticoccidian medication, Toltrazuril. Toltrazuril leads to a reduction of enzymes of the respiratory chain of the parasites and it is efficient against all intracellular developmental stages of Eimerian coccidians. At the same time, it does not interfere with development of natural immunity (Darius *et al.*, 2004; Mathis *et al.*, 2003). Subsequently, treatment group was infected orally with sporulated oocysts, while the control group received another round of medication in order to increase differences in infection intensity between control and infected birds. Infection intensities never rose to pre-medication level, presumably because of the lasting effects of pre-experimental treatment with Toltrazuril. Therefore, our experiment enabled us to assess the physiological impact of a mild infection.

During the peak phase of infection, infected greenfinches had on average 8% higher levels of plasma MDA than uninfected birds. It is most likely that higher plasma MDA levels in infected birds indicate oxidative damage generated during the immune response against Isosporan infection. It has been shown that Eimerian infection in domestic chicken induces macrophages to produce superoxide radical (Allen, 1997) and nitric oxide (Lillehoj & Li, 2004), which are toxic to parasites (Min & Lillehoj, 2004). Infection did not reduce body mass, plasma triglycerides and carotenoids, which reinforces the conclusion that oxidative damage primarily resulted from the immune responses rather than from malabsorption of dietary antioxidants. To my knowledge, this is the first evidence of an association between coccidian infection and oxidative damage in a wild animal species. The fact that plasma MDA was the single biochemical variable that responded to experimental infection with generally mild consequences is in accordance with the relevance of the potential immunopathological impact of oxidative stress in ecological context.

### **3.6. Oxidative stress and information content of black and yellow plumage coloration**

Carotenoids and melanins are two main pigment classes responsible for the colorful plumage of birds. Both types of pigments are claimed to be associated with oxidative stress (Griffith *et al.*, 2006; McGraw, 2005; Moreno & Møller, 2006), although the mechanisms of these links are largely unknown, and in case of melanin, also relatively poorly studied. It has been hypothesized that because a key intracellular antioxidant – glutathione (GSH) – inhibits eumelanogenesis, eumelanin-based black ornaments signal the bearer’s ability to cope with oxidative stress (Galván & Alonso-Alvarez, 2008). In this study, I addressed the question about the links between oxidative damage, systemic GSH levels, and melanin- and carotenoid-based feather coloration in captive greenfinches. I tested whether low systemic GSH levels increase and dietary carotenoids decrease oxidative damage to cell membranes by measuring malondialdehyde (MDA) levels. Additionally I tested the effects of manipulating carotenoid and GSH levels on the birds’ plumage coloration. Finally, I asked if it is possible to establish any links or trade-offs between carotenoid- and glutathione levels, and in formation of intense yellow (carotenoid-based) and black (eumelanin-based) coloration.

To manipulate carotenoid- and GSH-levels, a 2x2 factorial experiment was performed. Half of the birds received carotenoids in their drinking water and half of the birds were injected with BSO, a synthetic amino acid that inhibits gamma-glutamylcysteine synthetase, thereby depleting cells of GSH. Treatment with BSO suppressed erythrocyte GSH and led to an increase in plasma MDA by 14%. Birds with the highest drop in erythrocyte GSH level generally also had the highest increase in plasma MDA level and lost more weight during the experiment. Loss of body mass can be considered one of the most sensitive indicators of physiological condition in avian studies, which suggests that increased plasma MDA levels in our study indeed reflect unfavorable changes in health state. In addition to increasing oxidative damage, BSO treatment also decreased brightness of black feather tips, presumably by increasing eumelanin synthesis. This result implies that GSH may indeed be one of the mechanisms that maintain eumelanin ornaments as honest signals of quality. According to the honest signaling principle, because GSH depletion implies costs, individuals of low quality would not be able to invest in a eumelanin signal as much as high quality individuals.

As expected, carotenoid supplementation enhanced the chroma of tail feathers regrown during the experiment and there was a strong correlation between plasma carotenoid levels and feather chroma. This implies that carotenoid-based plumage coloration may indeed reflect the bearer’s ability to obtain carotenoid-rich food from the environment. As for the hypothesis stating the antioxidant role of carotenoids, the current study fails to support this idea. Contrary to my expectations, carotenoid supplementation did not affect plasma

MDA levels. Neither did manipulation of GSH affect blood carotenoid levels, which would be expected if carotenoids mirror the availability of other antioxidants (as proposed by Hartley and Kennedy (2004)). This result seems compatible with the review by Costantini and Møller (2008), finding little support for antioxidant function of carotenoids in birds. However, most of the studies reviewed in this article used methods that may not reveal the whole extent of antioxidant potential of carotenoids. Thus, so far, there is no sufficient information for either supporting or rejecting the possible antioxidant function of carotenoids in adult birds. This question has to be addressed in further experiments involving manipulation and assessment of different antioxidant systems.



## SUMMARY

Immunoecology is a demanding field of science: binding together two seemingly remote disciplines of ecology and immunology is a challenge. There is no consensus over what immunological (or other) methods would be best for answering ecological questions. Therefore, solving ecological problems must be carried out simultaneously with working out and refining methods, mechanisms and model systems suitable for this multidisciplinary approach. Accordingly, this thesis contributes to the emerging field of immunoecology in two ways: first, it tries to find out and validate methods and model systems suitable for immunoecological studies, and second, it applies these methods to answer some of the most actual questions in this area of research.

First, I tested the long term impact of classical immunoecological technique, subcutaneous injection of PHA, on leukocyte profiles of greenfinches. I found that PHA causes prolonged heterophilia. Although this does not necessarily mean that PHA causes serious long term health impact, this knowledge is important when PHA skin test is applied in studies monitoring changes in individual physiological condition.

Immunoecology is becoming more and more intertwined with studies of oxidative stress ecology. To answer the questions about the role of oxidative stress in immunoecology, it is crucial to find out suitable biomarkers of oxidative status. Accordingly, I described the covariation and consistency of eight widely used indexes of oxidative status. Three of the studied parameters showed individual consistency over 8 day period, but none of the parameters correlated significantly with each other. This might imply that all of the studied parameters contain unique information about the organism's physiology. At the same time, it is possible that the experimental conditions were too mild for any associations to emerge. Therefore, further studies of the parameters of oxidative status in wild animals would benefit from the experimental induction of OS.

Proceeding with the methodological side of my thesis, I tested the suitability of captive greenfinches for ecophysiological research. Since we are still in the early stages of the development of immunoecology as an independent field of science, development and adjustment of novel methods is an important part of the work in this area, and this work is mainly carried out in laboratory conditions. In order to be able to generalize the results obtained from captive animals to natural situations, it is crucial to understand how captivity *per se* affects different health state indexes. I studied the effect of captivity on 12 physiological parameters and found that although some of the parameters seemed to be affected by captivity (mainly indexes that were affected by diet), most were not, and therefore, physiological data collected from captive birds should be valid in other situations as well.

Next, I applied the captive greenfinch model to study one of the most intriguing questions in immunoecology – the possible link between carotenoid-based ornamentation and immune function. I assessed the effect of carotenoids

on the immune system on functional level, observing the changes in chronic coccidiosis infection intensity. A novel aspect of the study involved examination of the role of carotenoids in immune response through controlled immune suppression and immune activation experiments. Immune suppression with a synthetic stress hormone confounded the effects of immune activation and carotenoid supplementation. This finding implies that stress (and possibly variable stress reactivity of different individuals) is a potentially confounding factor in studies trying to assess the role of carotenoids in immune response. Among birds not treated with immune suppressant, immune activation suppressed infection, but only among carotenoid-supplemented birds. The findings about involvement of carotenoids in modulation of immune response against coccidiosis suggest that such research has great potential in explaining why yellow, orange and red animal colouration has evolved.

The greenfinch coccidiosis model is also applicable for investigating the associations between immune function and oxidative stress. When infection levels were experimentally manipulated, it came clear that coccidian infection causes oxidative damage in greenfinches. Since coccidians themselves do not generate reactive species in the course of infection, it is most probable that oxidative damage resulted from the immune responses of the host. This finding supports the understanding that immune responses are costly and that these costs may arise primarily as accompanying tissue damage. This study is the first evidence of an association between coccidian infection and oxidative damage in a wild bird species.

Since the plumage of greenfinches contains both carotenoid- and melanin-based ornaments, this species can also be applied as a model for studying the mechanisms that keep signal traits honest. Both types of pigments are claimed to be associated with oxidative stress, either as antioxidants, or as indicators of the levels of other antioxidants. By manipulating the levels of dietary carotenoids and glutathione (a key intracellular antioxidant that suppresses the production of the black pigment eumelanin), I tested the associations between plumage ornaments and oxidative status of the birds. Suppressing glutathione levels resulted with oxidative damage to cell membranes, simultaneously increasing the darkness of black feather tips. This finding implies that since GSH depletion is costly in terms of oxidative damage, only high quality individuals can afford to produce dark feather tips. Eumelanin-based plumage might therefore serve as an honest signal of individual quality. At the same time, although dietary carotenoid supplementation enhanced the chroma of yellow feather patches, it did not prevent oxidative tissue damage. This study fails to support the idea of carotenoids as active antioxidants. Despite decades of intense work, the question what exactly is being signaled by carotenoid ornaments is still very much open.

In conclusion, studying captive greenfinches seems to be a promising approach for answering many intriguing questions in immunoecology. Their good captivity tolerance allows studying a wild species in controlled laboratory

conditions. Their plumage ornaments allow studying honesty of signal traits. Their common infection with coccidians is easy to follow and manipulate, making them good models for studying the costs of immune activation and the associations between immune system and other traits. At the same time, although some model systems and methods might seem to be widely accepted and technically completed, work with old and new methods must always continue in order to find possible flaws, better interpretation possibilities and new solutions. Continuous methodological work will eventually help to bind together immunology and ecology, and this synthesis will enable scientists to find solutions to many questions that both disciplines separately would fail to answer.

## SUMMARY IN ESTONIAN

### **Rohevintide hematoloogilised tervisenäitajad: individuaalse varieeruvuse põhjused ja vastused immuunsüsteemi manipuleerimisele**

Individuaalne varieeruvus vastuvõtlikkuses haigustele on väga suur. Selle varieeruvuse põhjuste väljaselgitamine on viimasel ajal muutunud võtmeküsimuseks, kuna on selge, et parasiitide rolli evolutsioonilise jõuna ei tasu alahinnata. Parasiitne eluviis on väga levinud – pole olemas liiki, kes poleks parasiitidega nakatunud, ning paljud parasiidid on liigispetsiifilised. Sellest järeldub, et valdav enamus Maal elavatest liikidest on parasiidid. Et sellise mitmekesise ründajate hulga toime tulla, on organismidel välja arenenud võrdväärsetl keeruline ja mitmekesine kaitsemehhanism – immuunsüsteem. Nii immuunsüsteemi väljaarendamine, löögivalmis hoidmine kui ka rakendamine nõuab organismilt ressursse. Kuna looduses on ressursid enamasti piiratud, saavad parasiitidega võitlemiseks kulutatud ressursid (nii energeetilised kui ainelised) tulla vaid muude elutähtsate funktsioonide (nagu näiteks kasv, sigimine, oksüdatiivse stressi vastane kaitse, signaaltunnuste väljaarendamine) arvelt. See arusaam on pannud aluse uuele teadusharule – immuunökoloogiale. Immuunökoloogia eesmärgiks on välja selgitada mehhanismid, mis seovad immuunsüsteemi teiste elutähtsate funktsioonidega ja seletada selle kaudu looduslikes populatsioonides esinevat varieeruvust vastuvõtlikkuses haigustele. Nende eesmärkide saavutamiseks kasutatakse selles multidistsiplinaarses teadusharus meetodeid nii immunoloogia, ökoloogia, parasitoloogia, füsioloogia kui ka mitmete teiste teadusharude arsenalist.

Kui vaadelda immuunsüsteemi kogu organismi kontekstis, arvestades ka teiste elutähtsate funktsioonidega, võib aru saada, miks organismid haigustega võitlemiseks alati maksimaalset pingutust ei tee ja miks vastuvõtlikkus haigustele populatsioonide siseselt nii suures ulatuses varieerub. Immuunvastusel on oma hind ning see, kui suures osas organism on valmis või võimeline seda hinda maksma, võib oleneda väga erinevatest organismisisestest ja -välistest teguritest. Kuigi immuunvastuse hinna olemasolu on praeguseks valdavalt aktsepteeritud, pole veel kaugeltki selge, milles see hind väljendub ja millised mehhanismid immuunvastuse hinna teiste tunnustega seovad. Kui varasemad uurimused peavad immuunvastuse hinnaks eelkõige energia- ja toitainete kulu, siis uuemates töödes on välja pakutud oksüdatiivsete kahjustuste hüpotees. Selle hüpoteesi kohaselt on kulukas eelkõige immuunvastuse rakendamine – parasiite hävitades paiskavad immuunsüsteemi rakud välja reaktiivseid osakesi, mis valimatult kahjustavad nii sissetungijaid kui ka organismi enda rakke. Immuunvastuse hinna mõõtmine on aga keeruline ülesanne, mida raskendavad nii puudulikud teadmised immuunsüsteemi tööst kui ka raskused sobivate meetodite leidmisel organismile realselt kuluka oksüdatiivse stressi taseme hindamiseks.

Oksüdatiivne stress on olukord, kus reaktiivsete osakeste ja neid kahjutuks muutvate antioksidantide tasakaal organismis on paigast ära ja see viib koekahjustuste tekkele. Oksüdatiivne stress võiks olla üks mehhanismidest, mis vahendab erinevaid elukäiguomaduste ja signaaltunnustega seonduvaid lõiv-suhteid, kuid selle hüpoteesi tõestamiseks on vaja usaldusväärseid meetodeid oksüdatiivse stressi mõõtmiseks. Selliste meetodite väljatöötamine ja kontrollimine ökoloogiliste uuringute mudelsüsteemides on käimasolev ja aktuaalne protsess.

Loomaökoloogias on linnud kujunenud peamisteks mudelorganismideks oksüdatiivse stressi uurimisel. Linnu-uurijate suur huvi oksüdatiivse stressi vastu võib tuleneda hüpoteesist, mille kohaselt linnu oksüdatiivne staatus mõjutab tema sulestikuornamentide väljakujunemist. Neid ornamente kasutatakse signaaltunnustena nii paarilise valikul kui ka sotsiaalse hierarhia paikapanemisel. Peamised lindude sulestikus kasutatavad pigmendid on karotenoidid ja melaniinid, mis mõlemad võivad olla seotud oksüdatiivse stressiga. Toidust saadavad pigmendid karotenoididel osalevad väidetavalt nii immuunsüsteemi töös kui ka oksüdatiivse stressi ennetamises, kuigi selged tõendid mõlema funktsiooni kohta puuduvad. Melaniini tootmine toimub organismisiselt ning võib iseenesest olla reaktiivseid osakesi tekitav protsess, lisaks võib ka melaniinil olla antioksidantseid omadusi. Uue hüpoteesi kohaselt sõltub melaniini tootmine ka kõige levinuma rakusisese antioksidandi glutatiooni tasemest.

Käesoleva töö eesmärkideks oli (1) kontrollida mõnede laialt kasutusel olevate meetodite sobivust ning rakendamisvõimalusi immuunökoloogilistes uuringutes ning (2) leida vastuseid viimasel ajal kerkinud aktuaalsetele immuunökoloogilistele küsimustele. Töö esimeses pooles uurisin lindude immuunökoloogias immuunsüsteemi töö hindamiseks laialt kasutusel oleva meetodi (PHA süstimine) pikajalisi mõjusid, erinevate oksüdatiivse staatuse parameetrite ajalist varieeruvust ning omavahelisi korrelatsioone ning vangistuses elavate rohevintide sobivust immuunökoloogiliste uuringute jaoks. Töö teises osas uurisin karotenoidide rolli immuunvastuse reguleerimises, immuunvastuse oksüdatiivset hinda ning karotenoidsetes ja melaniinsetes sulestikuornamentides sisalduvat informatsiooni. Oma töös kasutasin mudelorganismina loodusest püütud rohevinte.

Kuigi fütohemaglutiniini (PHA) süstimise tulemusena tekkiv paistetuse on juba aastakümneid laialt kasutusel rakulise immuunsuse efektiivsuse hindamiseks, pole selle meetodi pikaajalist mõju lindudele uuritud. Artiklis 1 uurisin PHA süstimise mõju 3 ja 30 päeva peale manipulatsiooni. Tervisenäitajatena kasutasin leukotsüütide kontsentratsiooni veres. Nende abil saab lindudel hinnata nii immuunsüsteemi aktiveerimist kui ka stressitaset. Leidsin, et PHA süstimine põhjustas pikaajalise heterofiilide (kaasasündinud immuunsüsteemi mitespetsiifilised rakud) kontsentratsiooni tõusu, mis võib viidata kauakestvale põletikureaktsioonile. Selline seisund võib olla organismile kahjulik, kuna heterofiilide poolt välja paisatud reaktiivsed osakesed kahjustavad ka organismi

enda kudesid. Seda tulemust tuleb edaspidi arevsse võtta, kui PHA-testi soovatakse kasutada pikemaajalistes loomade konditsiooni hindavates uuringutes.

Teises artiklis uurisin mõnede laialt kasutusel olevate oksüdatiivse staatuse näitajate ajalist korduvust ning omavahelisi korrelatsioone. Organismi oksüdatiivse staatuse hindamiseks oleks ideaalne kasutada võimalikult paljusid erinevaid näitajaid, et saada sellest ülimalt keerulisest ja mitmetahulisest süsteemist adekvaatne pilt. Samas on loomalt võetavate proovide hulk alati piiratud, eriti loomaökoloogia mudelorganismideks olevatel loomadel, kes on enamasti väiksed ja kelle surmamist katse käigus tuleks vältida. Seetõttu on hädavajalik töötada välja näitajate kogum, mis annaks võimalikult ülevaatliku pildi organismi tööst, kuid mille hulgas poleks korduvaid ja samasisulisi näitajaid. Mõõtsin kaheksat erinevat parameetrit, mille hulgas oli nii üksikuid antioksidante, indekseid plasma üldise antioksidantsuse mõõtmiseks kui ka üks oksüdatiivsete kahjustuste marker. Ükski uuritud näitajatest omavahel ei korreleerunud, mis viitab, et kõik mõõdetud parameetrid sisaldavad unikaalset informatsiooni ning rõhutab veelkord ohtu üle tõlgendada üksikuid mõõdetud parameetreid ja vajadust mõõta võimalikult paljusid parameetreid. Kolm mõõdetud parameetrit olid lühema ajaperioodi jooksul individuaalselt püsivad, mis näitab nende parameetrite sobivust isendite-vaheliste erinevuste hindamiseks. Pikemaajaline korduvus ajas puudus kõigil mõõdetud parameetritel. Seoste puudumine ja vähene ajaline korduvus võisid osaliselt tuleneda ka valimimahtudest, mis võimaldasid tuvastada vaid tugevaid seoseid.

Kuigi ökoloogilisest aspektist oleks ideaalne uurida loomi nende looduslikus elukeskkonnas, muudab organismi looduses mõjutavate tegurite rohkus selliste uuringute tõlgendamise keeruliseks. Alternatiiviks on uurida looduslike liike vangistuses, kuid selleks tuleb olla teadlik vangistuse mõjust loomade tervisele. Kolmandas artiklis uurisin, kuidas mõjutab vangistus rohevintide tervisenäitajaid, võrreldes vangistuses elavaid linde vabalt elavate lindudega. Kuigi vangistus mõjutas mõnesid uuritud parameetreid (eelkõige looduses ja vangistuses erinevast dieedist tulenevaid näitajaid, mida on edaspidistes uuringutes võimalik reguleerida dieedi täiendamisega), oli enamiku parameetrite tase looduses ja vangistuses sama. Kõige olulisema näitajana ei erinenud stressitaseme indikaator vangistuses ja looduses. Samas esines mõnedes parameetrites ööpäevane varieeruvus, mistõttu proovide võtmise kellaaega tuleb edaspidistes uuringutes kindlasti arvesse võtta. Üldjoontes kinnitas see uuring vangistuses elavate rohevintide sobivust immuunökoloogilisteks uuringuteks.

Seoseid karotenoidide ja immuunvastuse vahel on uuritud mitmetes teadustöodes ning tulemusi on saadud nii- ja naasuguseid. Enamikus neist on kasutatud kunstlike antigene, mis ei pruugi peegeldada loodusliku olukorda. Neljandas artiklis uurisin karotenoidide lisatoitmise mõju kroonilisele koktsiidnakkusele. Kuna organismisisesed lõivsuhted ja seosed tulevad sageli esile vaid rasketes oludes, rakendasin karotenoidide rolli uurimiseks kahte lisamanipulatsiooni. Süstides stressihormooni analoogi (deksametasoon, DEX), simuleerisin stressist tingitud immuunsupressiooni. Süstides PHA-d, sain testida

immuunsüsteemi eelneva aktiveerimise mõju. DEX-i süstimine surus immuunsüsteemi töö alla ning muutis segaseks PHA ja karotenoidide mõju nakkusele. Kui DEX-iga süstitud linnud kõrvale jätta, selgus, et immuunsüsteemi aktiveerimine PHA-ga surus nakkuse alla, kuid ainult karotenoidide saanud lindudel. Lisaks vähendas karotenoidide lisatoitmine immuunaktiivsioonist põhjustatud kaalukadu. Need tulemused näitavad, et karotenoididel võib tõepoolest olla roll immuunvastuse ja selle hinna kujunemisel, kuid seda rolli mõjutavad mitmed organismisisesed tegurid, mistõttu karotenoidide rolli uurimine immuunvastuses on teadlastele tõsiseks väljakutseks.

Immuunvastusest tulenevad oksüdatiivsed koekahjustused võivad moodustada olulise osa immuunvastuse hinnast. Neljandas artiklis uurisin, kas koktsiidinakkus tekitab rohevintidele oksüdatiivseid kahjustusi. Sellele küsimusele vastamiseks manipuleerisin nakkuse taset, manustades lindudele koktsiidivastast ravimit ning nakatades ühte gruppi seejärel koktsiididega. Kuna nakkuse intensiivsus ei tõusnud ravieelsele tasemele, võib eeldada, et tegemist oli suhteliselt leebe nakkusega. Leidsin, et juba selline nakkus tekitas oksüdatiivseid koekahjustusi. Kuna koktsiidid ise peremeeste ründamiseks reaktiivseid osakesi ei kasuta, tekitasid kahjustusi ilmselt koktsiidide vastu võitlevad immuunsüsteemi rakud. Lindudel, kellel oli rohkem kahjustusi, õnnestus koktsiidinakkuse allasurumine paremini. See tulemus näitab, et oksüdatiivsed kahjustused moodustavad tõepoolest osa immuunvastuse hinnast.

Kui sulestikuornamendid näitavad linnu kvaliteeti, peavad esinema mehhanismid, mis tagavad nende signaalide aususe – kõige uhkemaid ornamente saavad toota vaid kõige kvaliteetsemad isendid. Karotenoidsete ja melaniinsete signaalide aususe tagamiseks on välja pakutud erinevaid hüpoteese. Karotenoidsed ornamendid peegeldavad muuhulgas väidetavalt ka linnu oksüdatiivset staatust – vaid need linnud, kes ei pea karotenoidide kasutama antioksidantidena, saavad need pigmendid paigutada sulgedesse. Melaniinsed ornamendid aga näitavad uue hüpoteesi kohaselt linnu võimet tulla toime oksüdatiivse stressiga, kuna peamise rakusisese antioksidandi glutatiooni (GSH) kõrge tase surub mustade eumelaniinsete pigmentide tootmist alla. Nende hüpoteeside kontrollimiseks manipuleerisin GSH ja karotenoidide taset lindude veres ja mõõtsin oksüdatiivseid kahjustusi. Glutatiooni allasurumine põhjustas oodatult oksüdatiivseid kahjustusi ning võimendas samal ajal eumelaniinseid sulestikuornamente, mis näitab, et mustad sulestikulaigud näitavad tõepoolest linnu võimet tulla toime oksüdatiivse stressiga. Karotenoidide manustamine muutis suled kollasemaks, kuid ei mõjutanud oksüdatiivseid kahjustusi. Selle uurimuse põhjal ei anna karotenoidsed ornamendid infot linnu võime kohta oksüdatiivse stressiga toime tulla.

Kokkuvõttes võib rohevinte pidada heaks immuunökoloogia mudelorganismiks. Nad taluvad hästi vangistust. Nende sulestikuornamendid võimaldavad uurida signaaltunnuste ausust tagavaid mehhanisme. Kõikidel meie töörühma poolt uuritud rohevintidel esinev krooniline koktsiidinakkus on kergesti jälgitav ja manipuleeritav, mis võimaldab selle mudelsüsteemi abil

uurida immuunvastuse hinna probleeme ning immuunsüsteemi seoseid teiste elutähtsate funktsioonidega. On selge, et koos immuunökoloogia võtmeküsimustele vastuste otsimisega peab paralleelselt toimuma ka meetodite testimine, uurimine ja väljatöötamine. Vaid nii on võimalik ühendada üksteisest pealtnäha kaugel asuvad distsipliinid – immunoloogia ja ökoloogia – ning nende teadusharude integreerimise abil leida vastuseid küsimustele, mida kumbki haru eraldi lahendada ei suudaks.



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## **PUBLICATIONS**

## CURRICULUM VITAE

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2010–2012 AS BIT, Publishing house Avita, Editor in the field of biology

2001–2009 Estonian Literary Museum, Editor in different projects

2003–2005 Teaduskeskus AHHA, Mentor, guide

### Publications

**Sepp, T.**, Karu, U., Blount, J., Sild, E., Männiste, M., Hõrak, P. (2012). Coccidian infection causes oxidative damage in greenfinches. *PLoS ONE*, 7 (5).

**Sepp, T.**, Sild, E., Blount, J., Männiste, M., Karu, U., Hõrak, P. (2012). Individual consistency and covariation of measures of oxidative status in avian blood. *Physiological and Biochemical Zoology*, 85 (3).

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Sild, E.; **Sepp, T.**; Hõrak, P. (2011). Behavioural trait covaries with immune responsiveness in a wild passerine. *Brain, Behavior, and Immunity*, 214, 3467–3473.

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Hõrak, P.; Sild, E.; Soomets, U.; **Sepp, T.**; Kilk, K. (2010). Oxidative stress and information content of black and yellow plumage coloration: an experiment with greenfinches. *Journal of Experimental Biology*, 213, 2225–2233.

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- Sarv, T.**; Hõrak, P. (2009). Phytohaemagglutinin injection has a long-lasting effect on immune cells. *Journal of Avian Biology*, 40, 569–571.

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### Publikatsioonide loetelu

- Sepp, T.**, Karu, U., Blount, J., Sild, E., Männiste, M., Hõrak, P. (2012). Coccidian infection causes oxidative damage in greenfinches. *PLoS ONE*, 7 (5).
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