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ELIN SILD

Oxidative defences in immunoecological context: validation and application of assays for nitric oxide production and oxidative burst in a wild passerine

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CONTENTS

LIST OF ORIGINAL PAPERS 6

1. INTRODUCTION 7
   1.1. Immunoecology and oxidative stress ecology 7
   1.2. Problems with understanding and assessment of immunity and oxidative stress 9
   1.3. Aims of the thesis 10

2. MATERIALS AND METHODS 13
   2.1. Study species 13
      2.1.1. Greenfinches 13
      2.1.2. Reactive oxygen species 13

3. RESULTS AND DISCUSSION 15
   3.1. Validation of methods: 15
      3.1.1. Assessment of nitric oxide production from blood plasma 15
      3.1.2. Assessment of oxidative burst in avian whole blood samples. 17
   3.2. Application of assays for nitric oxide production and oxidative burst 19
      3.2.1. Leptin, nitric oxide and immune responsiveness to phytohemagglutinin 19
      3.2.2. Connections between behaviour and immune responsiveness 21
      3.2.3. Carotenoids, immunity and oxidative defences 22

SUMMARY 24

SUMMARY IN ESTONIAN 26

REFERENCES 30

ACKNOWLEDGEMENTS 37

PUBLICATIONS 39

CURRICULUM VITAE 91

ELULOOKIRJELDUS 93

LIST OF ORIGINAL PAPERS

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

1. INTRODUCTION

The father of evolutionary biology, Charles Darwin, regarded parasites as fascinating examples of adaptation, but underestimated their role as an evolutionary force (Schmid-Hempel 2009). The significance of parasites as selective factors for a wide range of phenomena has only been studied in depth over the last few decades. This work has its roots in behavioural/evolutionary ecology and in population biology/ecology thus shaping a new comprehensive field of evolutionary parasitology (Schmid-Hempel 2009). Recognizing the evolutionary and ecological implications of selection pressure generated by parasites upon their hosts has offered novel explanations in domains such as sexual selection (Hamilton and Zuk 1982), the evolution of sex and recombination (Ebert and Hamilton 1996; Hamilton et al. 1990; Lively 2010), ethology (Moore et al. 2005; Thomas et al. 2005), life-history evolution (Schulenburg et al. 2009), and so forth.

Although it might seem obvious that it would always be best to fight off parasites and diseases fast and efficiently, hosts still remain susceptible to parasites and immune responses vary widely between individuals. In an attempt to explain why this variation exists and what are its consequences, a discipline of immunoecology has emerged in the last decade of XX century (Martin et al. 2011). This new field of research analyses immune defences in the context of ecology and adaptations in a framework of costs and benefits. (Schmid-Hempel 2003; Schmid-Hempel 2009; Schulenburg et al. 2009; Sheldon and Verhulst 1996).

Immunoecologists ask how immune defences have evolved and are being used and optimized in different environments, ecological settings and lineages. If immune responses require resources that could be potentially allocated to life-history traits such as growth and reproduction or sexual display, emergence of trade-offs between immune function and other components of fitness is inevitable (Lochmiller and Deerenberg 2000; Norris and Evans 2000; Sheldon and Verhulst 1996). From the ecological perspective, the well-being of an organism is maintained by efficiently matching biological and behavioral priorities to the demands of the environment. However, unlike some other organismal functions, the immune system is necessary for survival only when an immunological challenge such as infection is present (Segerstrom 2010). So it might be beneficial to down-regulate immune function if by using it the costs exceed the fitness benefits acquired.

1.1. Immunoecology and oxidative stress ecology

What are the costs of immune activation? Studies on different animal species have demonstrated that activation of the immune system can 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 French et al. 2009; Zuk and Stoehr 2002). However, the question about the currencies used for paying the costs of activation of immune defences has remained poorly understood. The traditional view of animal ecologists has been that the costs involved in life-history trade-offs are basically energetic (Stearns 1992), which is in good agreement with the high metabolic costs of febrile acute phase responses (e.g., Martin et al. 2008; Muehlenbein et al. 2010; Segerstrom 2010). On the other hand, it has been also claimed that energetic demands required for maintenance of immune function and for mounting specific immune responses are negligible (Klasing 2004; Klasing et al. 1999). Furthermore, experimental tests of these ideas have given contradictory results (reviewed by Burness et al. 2010; Lee et al. 2005; Nilsson et al. 2007). Therefore, an alternative hypothesis, proposing that costs of immune responses are primarily caused by the accompanying immunopathological tissue damages, is becoming increasingly popular (Dowling and Simmons 2009; Råberg et al. 1998; Sorci and Faivre 2009; von Schantz et al. 1999). Vertebrate innate immune system protects organism by producing reactive oxygen species in a process called oxidative burst. These oxygen species are highly reactive and destroy pathogens by damaging their proteins, lipids and DNA. These reactive species are not pathogen-specific and can also damage host tissues if there are not enough protective antioxidants present.

The use of reactive oxygen species in immune function connects immuno-ecology with another nascent discipline - oxidative stress ecology (McGraw et al. 2010). Oxidative stress is a situation when balance of pro-oxidants and antioxidants is shifted towards pro-oxidants and this causes oxidative damage to organisms' own tissues (Halliwell 2006; Sies 1997; von Schantz et al. 1999). Oxidative stress is believed to play important role in senescence, sexual ornamentation and sperm performance and can be linked with selection pressures to survival and reproduction (Costantini et al. 2010b; Metcalfe and Alonso-Alvarez 2010; Monaghan et al. 2009). Therefore, oxidative stress may appear one of the mechanisms that links immune function with other life-history traits (Costantini and Møller 2009; Sorci and Faivre 2009).

There are several similarities in the ecological approach to studying reactive oxygen species and immune function. In both cases researchers need measurable proxies for abstract concepts – immunocompetence or oxidative stress. The level of oxidative stress or immunocompetence cannot be determined by simply measuring one trait (Martin et al. 2006) – for instance the amount of some antioxidant or magnitude of antibody response, although this kind of approach has been used often (Bize et al. 2008; López-Rull et al. 2011; Møller and Erritzøe 2000). Both immunocompetence and oxidative status can only be assessed when measuring different components of these complex systems and even then caution is needed when interpreting the results. We must recognise that our knowledge about the physiology of living organisms is still quite primitive.

1.2. Problems with understanding and assessment of immunity and oxidative stress

Ecologists tend to treat organisms physiology as a black box and oversimplify this highly regulated and integrated system (Schmid-Hempel 2005). Our understanding of how nervous, endocrine and immune systems are coordinated remains incomplete, partly because the importance of the immune system as part of this regulatory network has only recently been recognized (Demas et al. 2011). Rather than acting independently of one another, these systems communicate in an integrated fashion to coordinate physiological and behavioural responses (Demas et al. 2011). Understanding the physiological mechanisms is crucial, as they have major impact on what questions can be asked and how the obtained results can be interpreted (Schmid-Hempel 2003; Schmid-Hempel 2005).

What makes describing these systems so complex? The answer lies in the nature of their functioning. While aiming at assessment of either immune function or oxidative damages, the major task for researcher is to distinguish between controlled (adaptive) and uncontrolled (non-adaptive) processes (Hõrak and Cohen 2010). Controlled processes result in physiological changes that are regulated in ways that tend to be advantageous to the organism, given its circumstances. Uncontrolled processes occur when the regulatory system is overwhelmed or does not exist, and are thus often not advantageous to the organism. For instance, cross-talk and regulatory/inhibitory mechanisms among immune defences have been described at several levels of immunity (Boughton et al. 2010; Demas et al. 2011). T-helper 1 (Th1) / T-helper 2 (Th2) polarization during infection imposes constraints upon an individual´s immune responsiveness, with the consequence that hosts cannot simultaneously mount strong responses using both Th1 and Th2 cells (Ardia et al. 2010; Hõrak et al. 2006; Mosmann and Sad 1996). Thus, it is important to consider assays as reflecting a component of immunological complexity and not as a general index of immunocompetence or individual quality, unless this can be explicitly demonstrated for the species of interest (Boughton et al. 2010).

All the same principles apply for the studies of oxidative stress. Variation in antioxidant levels does not necessarily mirror the variation in oxidative stress that the organisms experience. Costantini and Verhulst (2009) have explained that if researches measure only antioxidant capacity or some single class of antioxidants, they may interpret their results as showing oxidative stress, although the real situation might be far from this (Costantini and Verhulst 2009). For instance, high values of antioxidant capacity can reflect adaptive and compensatory responses to oxidative (or physiological) stress rather than optimal health condition (reviewed by Costantini et al. 2010a; Hõrak and Cohen 2010; Noguera et al. 2011).

Systematic individual differences add on to the challenge of measuring immune function and oxidative status. Even when several aspects of immune function of an organism are measured, the question whether decreased immune

response is maladaptive or if it might just be part of a different strategy or coping still remains. Considering organisms with stronger immune response or antioxidant defences as healthier might not always be justified. However, the initially prevailing paradigm inimmunoecology has been that 'immuno-competence' is an ubiquitous commodity, so that the stronger responses indicate the better health state (Boughton et al. 2010; Viney et al. 2005). The similar reasoning has been also widespread in early studies of oxidative stress ecology, i.e., high levels of plasma total antioxidant capacity have been interpreted as a sign of beneficial redox state. However, several experiments have demonstrated up-regulation of antioxidant capacity in response to oxidative challenge induced by either exhaustive exercise, immune activation or physiological stress (reviewed by Costantini et al. 2010a; Hõrak and Cohen 2010). Most likely, the optimal levels of both immune responses and antioxidant protection are different for individuals in poor and good condition. Healthy and resourceful individuals are expected to tolerate higher levels of immunopathological (Råberg et al. 1998; Sadd and Schmid-Hempel 2009) or oxidative (e.g., Hill 2011; Mougeot et al. 2009; Stier et al. 2009; Vinkler and Albrecht 2009) damage. Furthermore, assuming that the costs of immune response can out-weigh its potential benefits, one could easily imagine selection pressures for infection tolerance – an ability of host to reduce or alleviate the fitness loss owing to parasite infection, without reducing infection intensity or parasite growth (Baucom and De Roode 2010; Råberg et al. 2009). Thus, restricted immune responses and parasite tolerance may have evolved in order to limit the costs associated with immune defence (Graham et al. 2011).

1.3. Aims of the thesis

As outlined above, the recent developments in immunoecology are crucially dependent on the concept of the costs of immune responses, among those the collateral oxidative damage being one of the prime candidates. However, despite the prominence of the topic, attempts of animal ecologists to assess the importance of oxidative damages and antioxidant defences in vertebrate models have progressed slowly. Studies aimed at assessing whether immune system activation leads to oxidative damage have yielded contradictory results (Cohen et al. 2007; Costantini and Moller 2009; Hõrak et al. 2007; Sorci and Faivre 2009; Stier et al. 2009). Diversity of outcomes of those experiments can be probably partly ascribed to different study species, antigens, and experimental conditions. However, the difficulties in establishing the costs of immune activation also seem to relate to assessment methods of oxidative defences and interpretations of results. Some of those difficulties stem from specific limitations inherent to ecological research, including species specifity of many assays, requirements of non-lethality and often availability of only single time point measures and small sample volumes (Matson et al. 2005; Millet et al. 2007). Methodological progress in the field thus largely depends on the development

and validation of immune assays suitable for ecological studies. Consequently, the current thesis aims at elaboration, validation and application of two methods for assessment of production of reactive oxygen species during innate immune responses.

The first of these techniques concerns measurement of nitric oxide production from blood plasma. Nitric oxide (NO) is a modulator of inflammatory processes that also participates in killing parasites, virus-infected cells and tumor cells by formation of peroxynitrite, one of the most important initiators of the free radical damage. Uncontrolled production of NO can lead to nitrosative stress, causing damages to proteins, DNA and cell injury and death. Determination of NO production in animals is thus potentially informative about oxidative damages generated during immune responses, however, it's potential in ecophysiological research has remained almost totally unexplored (**Paper I**).

The second technique is an assay for measuring oxidative burst of avian phagocytic cells in response to bacterial antigen from the whole blood. Oxidative burst is a main effector mechanism of microbial killing by phagocytes. These cells destroy microbial organisms by producing reactive oxygen species (ROS), a process that is mediated by the enzyme NADPH oxidase. By validation and application of a Pholasin-based chemiluminescence assay (WBCL assay) my aim here was to provide a new tool for immunoecologists for quantification of reactive species produced during induced innate immune responses (**Paper II**).

Having validated the suitability of the above-mentioned assays for immunoecological research on small passerine birds, my next aim was to test their applicability for the basic research of some pertinent immunoecological problems. The first of those involves the question about the connections between the immune and endocrine systems. Leptin is a hormone and cytokine that informs an organism about availability of resources and is believed to be responsible for their proper reallocation between competing functions. Leptin and NO are both important messengers in intra- and intercellular communication systems in vertebrates. Several studies have demonstrated an involvement of both substances in the immune response. Here I tested the effects of chronic leptin and anti-leptin treatments on the NO production and phytohemagglutinin-(PHA) induced cutaneous inflammatory response in greenfinches, asking whether leptin administration increases systemic NO production and cutaneous inflammatory responses to PHA as shown in other taxa (**Paper III**).

In the next case study I addressed the question about the association between immune function and behaviour, applying the WBCL assay elaborated in Paper II for assessment of innate immunity in greenfinches with different degree of tolerance to captivity. Behaviour and immune responses are controlled by the neuroendocrine system, which is thought to result in covariation between personality types and stress- and immune-associated diseases. However, the relationships between behaviour and immunity have been almost exclusively studied in domestic or laboratory animals whose personalities have been systematically altered in the process of domestication. On the contrast, next to

nothing is known about the associations between behaviour and immune traits in wild animals. Here I describe such an association in wild-caught greenfinches, showing that birds who tolerate captivity better, i.e., the individuals performing flapping flight bouts at lower frequency, mounted stronger antibody response to a novel *Brucella abortus* antigen and their circulating phagocytes were capable of producing stronger oxidative burst in response to stimulation with bacterial lipopolysaccharide *in vitro* (**Paper IV**).

In the final paper I investigated the associations between the immune-stimulated oxidative burst (WBCL response) and dietary carotenoids. Carotenoid-based coloration is used by animals in a variety of contexts, from mate attraction and agonistic displays to warning colourations and camouflage. It has been hypothesized that carotenoid colouration is used for communicating the health status of the bearers because carotenoids are efficient immunomodulators and/or antioxidants. However, the latter argument has been recently debated and the mechanisms by which carotenoids modulate immunity or oxidative balance are poorly known. Here I tested whether dietary carotenoid supplementation affects WBCL response and humoral immune response to *Brucella abortus* antigen. Additionally, I asked whether immune stimulation with bacterial lipopolysaccharide (LPS) affects blood carotenoid levels, as one might expect in the case of involvement of carotenoids in the immune response (**Paper V**).

2. MATERIALS AND METHODS

2.1. Study species

2.1.1. Greenfinches

Greenfinches (*Carduelis chloris*) are medium-sized (ca 28 g), sexually dichromatic gregarious seed-eating passerines native to the western Palearctic region. Males are larger and more colourful with sexually-selected (Eley 1991) yellow, carotenoid-based markings (Cramp and Perrins 1994; Saks et al. 2003a). Greenfinches tolerate captivity easily and have been used as a model of passerine species in ecophysiological research of relationships between ornamental traits and health status (Hõrak et al. 2004; Lindström and Lundström 2000; Merilä et al. 1999), and in numerous studies investigating the role of carotenoids as immunostimulators and antioxidants (Hõrak et al. 2007; Hõrak et al. 2010; Hõrak et al. 2006; Saks et al. 2003b; Sepp et al. 2011) and lately also in personality research (Herborn et al. 2011a; Herborn et al. 2011b).

2.1.2. Reactive oxygen species

Reactive oxygen species (ROS) is a collective term that describes all pro-oxidants derived from O_2. However there are also pro-oxidants derived from other elements such as nitrogen (reactive nitrogen species; RNS) (Halliwell and Gutteridge 1999). Reactive oxygen species (ROS) encompass a variety of diverse chemical species, including superoxide anions, hydroxyl radicals, hydrogen peroxide and nitric oxide radical (Finkel and Holbrook 2000; Sies 1993). The half-lives of the major reactive species are vastly different, for example hydroxyl radicals have diffusion limited reactions, i.e. they take place practically at the site of generation while some peroxyl radicals are relatively stable, with half-lives in the range of seconds (Sies 1993). These various radical species can either be generated exogenously or produced intracellularly from various sources (Finkel and Holbrook 2000).

ROS are generated as a result of normal intracellular metabolism in mitochondria and peroxisomes, as well as from a variety of cytosolic enzyme systems (Finkel and Holbrook 2000). Part of the immune system relies on immune cells that kill pathogens by releasing pro-oxidant compounds using a multi-component enzyme complex NADPH oxidase (Costantini and Møller 2009; de Oliveira-Junior et al. 2011).

Reactive species can accumulate to damage macromolecules, cells and tissues, but they also have important biological roles as regulatory agents in a range of biological phenomena (Murphy et al. 2011). Mechanisms of damage differ by cellular target and include lipid peroxidation, protein oxidation and nucleic acid oxidation (Halliwell and Gutteridge 1999). Organisms have evolved multiple defence lines to prevent oxidative damage, ranging from antioxidant enzymes to low molecular weight antioxidants, and also specific

cellular components that repair oxidatively damaged molecules (Costantini and Verhulst 2009; Halliwell and Gutteridge 1999). A serious imbalance between the generation of reactive oxygen species and antioxidant protection in favour of the former, that causes excessive oxidative damage, is referred to as oxidative stress (Halliwell 2011; Sies 1997).

The occurrence of oxidative stress has been implicated in numerous pathologies and it has also been suggested that oxidative stress may play an important role in the ageing process (Finkel and Holbrook 2000; Freinbichler et al. 2011). Ecologists have tried to tackle the free-radical theory of ageing by comparing ROS production and lifespan in different animal species and this has yielded in contradictory results (Buttemer et al. 2010; Speakman and Selman 2011). Oxidative stress has also been shown to influence many other important aspects of fitness, such as sperm performance and sexual ornamentation, and altogether it is evident that antioxidant availability and oxidative stress can be ecologically relevant selection pressures linked to organismal survival and reproduction (Costantini et al. 2010b).Therefore it is not surprising that among newest blossoming ecological areas are sister fields of antioxidant ecology and oxidative-stress ecology (McGraw et al. 2010) that try to explain variation in individual fitness and life-history traits by oxidative balance (Monaghan et al. 2009) and add to the comprehensive understanding of the complexity of oxidative balance systems by the knowledge of specificity and generality of results across species and conditions (Cohen et al. 2010).

3. RESULTS AND DISCUSSION

3.1. Validation of methods:

3.1.1. Assessment of nitric oxide production from blood plasma

Paper I describes a simple assay for determining the production of nitric oxide (NO), a multifunctional signalling and effector molecule with diverse physiological functions. The suitability of this assay for assessment of individual variation of physiological condition and activation of phytohemagglutinin-induced immune response in captive greenfinches was also tested.

Nitric oxide is biosynthesized by oxidation of L-arginine by nitric oxide synthase (NOS), an enzyme with three isoforms. These isoforms are differentially expressed and regulated in various tissues and cells, allowing selective control and site-directed activity of NO when it is generated (Vajdovich 2008). Neuronal isoform of NO synthase (nNOS) produces NO in nervous tissue where it governs various functions such as apoptosis, differentiation, development and synaptic plasticity (Vajdovich 2008). Endothelial nitric oxide synthase (eNOS) generates NO in blood vessels and heart where its main role is to control blood pressure by reduction of basal vascular tone and mediation of flow-induced vasodilation.

While the synthesis of nNOS and eNOS is constitutive, inducible nitric oxide synthase (iNOS) activity is regulated predominantly at the level of gene transcription. Its expression is absent or very low in resting cells but is markedly increased when induced by inflammatory cytokines, microorganisms, endotoxins, hypoxia and reactive oxygen species. Induction is robust, and NO generated from the induced enzymes is about three orders of magnitude higher than that generated from constitutive NOS (Wu 2001).

NO participates in controlling the pro- vs. anti-inflammatory phenotype of macrophages and therefore has an important regulatory role in inflammatory process. Induced NO production is one of the principal mechanisms of macrophage cytotoxicity for tumor cells, bacteria, fungi, protozoa and helminths (Bogdan et al. 2000; Victor et al. 2004). Excessive, prolonged production of NO contributes to tissue damage in septicemia, ischemia-reperfusion injury, arthritis, and other inflammatory conditions (Vajdovich 2008; Victor *et al.* 2004). All this suggests that measuring NO production has especially promising role in immunoecological research, such as assessment of the magnitude of inflammatory response and potential immunopathological damages. Indeed, NO production assay has been at least once used as a proxy of measurement the activation of innate immunity in an ecological study (Bourgeon et al. 2007). Yet much more frequently, NO production has been measured in poultry studies as a convenient index for assessment of the severity or pathogenesis of intestinal coccidiosis (e.g., Allen 1997; Allen and Fetterer 2000; Lillehoj and Li 2004; Zhu et al. 2000)

The simple method for measuring NO production via determination of nitrites and nitrates has three main steps. The first is deproteinization of plasma, then followed by nitrate reduction to nitrite with activated cadmium granules and last step is Griess reaction that measures the concentration of nitrites. Chemical deproteinization used in this assay is much cheaper and faster compared to alternative ultrafiltration, but dilutes sample. Cadmium-based nitrate reduction used in this assay gives larger linear measurement range compared to enzymatic methods.

Individual NO_x values were moderately correlated when measured over 6-day interval. This implies that NO production in captive greenfinches reflects relatively short-term changes in individual physiology. Birds injected with phytohemagglutinin had on average 21 % higher NO levels at the third day after treatment as compared to the saline-injected birds. Most likely explanation for this is inflammation-induced NO production. Carotenoid or dexamethasone treatments did not affect NO production. Because glucocorticoid hormones suppress NO production (Vajdovich 2008), we expected lower NO_x concentrations in birds treated with synthetic corticosteroid dexamethasone. This, however was not the case although dexamethasone treatment efficiently suppressed PHA-induced swelling (Sepp et al. 2011). No sex differences were found in NO levels among wild greenfinches. Wild greenfinches had on average 31% lower NO production than those who had spent 26 days in captivity by the time of sampling. Individual changes in coccidian infection intensity between two blood-sampling periods did not predict corresponding changes of NO levels. In chickens experimentally infected with Eimerian coccidians, NO production appeared an excellent marker of the severity of inflammatory response as it correlated positively with infection dose and negatively with body mass gain (Allen and Fetterer 2000). Absence of correlations between coccidian infection intensity and NO production in the current study can be probably ascribed to the chronic phase of infection in greenfinches as compared to the acute phase in chicken experiments. One might thus speculate that individual differences in intensity of coccidiosis mainly contribute to inter-individual variation in NO production in the acute but not chronic phase of infection. This hypothesis has to be tested in infection experiments.

In conclusion, results of this study suggest that determination of NO production from blood is likely to offer novel information for ecological animal studies. The assay protocol is simple, accurate, and inexpensive, and small amounts of blood required make it applicable for a wide range of model species such as passerine birds and small reptiles or mammals.

3.1.2. Assessment of oxidative burst in avian whole blood samples

In paper II a novel Pholasin-based chemiluminescence method for assessment of oxidative burst in the whole blood samples of birds is validated and applied. This assay measures an inducible component of innate immunity by quantifying the immediate extracellular oxidative burst of stimulated phagocytes.

Oxidative burst is a main effector mechanism of microbial killing by phagocytic cells. These cells destroy microbial organism by producing reactive oxygen species (ROS), a process that is mediated by the enzyme NADPH oxidase (Freitas et al. 2009). The primary product of this oxidase is superoxide anion (O_2^-) which interacts with other molecules to generate more noxious reactive species such as peroxynitrite and hydroxyl radicals and hypochlorous acid. These compounds exhibit a broad spectrum of biotoxicity and thus form a critical component of innate immunity, serving as the first line of defence against microbial infection.

Research on ROS production by avian leukocytes has been predominantly performed on separated heterophils (He et al. 2007) or macrophages (Chadfield and Olsen 2001). Due to the limitations of the available amount of blood, such assays cannot be applied to typical study objects of ecologists such as passerine birds and small reptiles and mammals. Also the functional state of whole blood phagocytes may reflect the physiological state of the host better than that of isolated leukocytes because isolation of cells can alter the viability, activity and receptor expression of cells (Papp and Smits 2007). Only few reports exist on the application of the WBCL response on avian blood (Papp et al. 2009) and these studies (as well as studies on isolated cells) have measured ROS production during 0.5–3 h after induction of oxidative burst where the luminescent signal is mainly generated by production of ROS during degranulation of heterophils.

Oxidative burst in the whole blood samples was measured using ABEL® Cell Activation test kit with Pholasin® and Adjuvant-K™ (Knight Scientific, Plymouth, UK). Pholasin is a 34 kD photoprotein from the bioluminescent mollusc *Pholas dactylus* that emits light in the presence of reactive molecules such as superoxide anion, singlet oxygen, hydroxyl and ferryl radicals, nitric oxide, hypochlorous and hypobromous acids, chloramines, bromamines, peroxynitrite and peroxidases. The assay was originally designed for human blood and the originally recommended stimulants of oxidative burst are fMLP (n-formyl-methionyl-leucyl-phenylalanine), which binds to specific receptors and PMA (phorbol-12-myristate-13-acetate), which activates NADPH oxidase independently of receptors and stimulates degranulation of phagocytic cells. Chicken heterophils lack the receptors for fMLP (Gerilechaogetu et al. 2009; Kogut et al. 1998) and this finding was corroborated for greenfinches, as the luminescence did not increase in response to fMLP injection.

For adjusting the Pholasin-based assay for avian blood I used high concentrations of bacterial lipopolysaccharide (LPS) as a stimulant. WBCL responses induced by injection of bacterial LPS into avian whole blood samples

were rapid (in seconds, see figures 2–5 in Paper II), implying that unlike the previously used stimulants, LPS induced an immediate extracellular oxidative burst of phagocytes. At high concentrations of the stimulant, the magnitude of increase of luminescence signal was more than 200 times over baseline. To my knowledge, this strong increase in luminescence signal has been never obtained by using traditional optical probes. Another methodological advancement was using consecutive *in vitro* injections of a stimulant with a two-minute interval. In general, the secondary response was higher than the primary one which indicates that phagocytic cells in the blood can be primed to produce more ROS shortly after the first encounter with a stimulant. The relative magnitude of this priming effect is captured by the parameter peak ratio (maximum of the secondary response / maximum of the primary response). Interestingly, this parameter showed highest individual consistency over the six-day period and also correlated most strongly with concentrations of heterophils and lymphocytes after *Brucella abortus* inoculation *in vivo*. I am not aware that such a "potentiation ability" of phagocytes has been studied before in birds, but this study suggests that this variable may capture biologically interesting information.

Blood of all six studied passerine species produced chemiluminescence response to stimulation with bacterial LPS *in vitro*. The magnitude of the response depended on the concentration and origin of the LPS. Parameters of this response depended on biological factors such as age of the birds and *in vivo* priming with different antigens such as LPS and *Brucella abortus* antigen suspension. Repeatabilities of all parameters of the WBCL response were significant, but not particularly high for some measures such as baseline luminescence.

In conclusion, this study demonstrated that a Pholasin-based assay for assessment of WBCL response is suitable for application in birds. It could be specifically useful in comparative immunoecological research (Hasselquist 2007), but also in answering questions about how dietary antioxidants (Olsson et al. 2009; Walrand et al. 2005) and other compounds (McReynolds et al. 2009) or manipulation of physical activity and infection affect ROS production during innate immune responses. Equally interesting would be to test whether individuals with higher WBCL response appear more resistant to experimental infections or more susceptible to oxidative stress.

3.2. Application of assays for nitric oxide production and oxidative burst

3.2.1. Leptin, nitric oxide and immune responsiveness to phytohemagglutinin

Paper III tested the effects of chronic leptin and anti-leptin treatments on the NO production and phytohemagglutinin (PHA) induced cutaneous inflammatory response in greenfinches. Leptin is a peptide hormone that is mainly produced by white fat-cells and released into the circulatory system (Gertler 2006). Normally, the plasma concentration of leptin is proportional to the amount of adipose tissue in most species. An increase in the level of leptin signals the hypothalamus to activate a negative feedback loop that usually leads to a reduction in food intake and engagement in other activities (Gertler 2006; Lõhmus et al. 2004). Leptin´s role as an important metabolic signal mediating many physiological functions has been documented in many vertebrate species including rodents, farm animals, humans and some domesticated and wild birds (Gertler 2006; Kordonowy et al. 2010; Lõhmus et al. 2003; Ohkubo and Adachi 2008; Quillfeldt et al. 2009).

Several studies have demonstrated an increase in circulating leptin levels during infection and inflammation, suggesting that leptin is part of the immune response and host defence mechanism (La Cava et al. 2004). From the life-history perspective, it is critical for organisms to divide energetic resources between processes with high energy demand without deleterious effects on survival. Therefore the role of leptin has likely evolved as a signal about the status of current energy supplies; this information may be transmitted, for example, to the hypothalamic-pituitary axis to regulate the costs associated with immune responses.

Leptin administration in rodents increases systemic NO production (reviewed by Sweeney 2002); however, whether this also applies to other taxa is unknown. The effects of leptin on systemic NO production have never been studied in birds, whose lipid metabolism differs from that of mammals. Here we tested the effects of chronic leptin and anti-leptin administration on the NO system in greenfinces. Additionally we investigated whether leptin administration affects a phytohemagglutinin- (PHA) induced cutaneous inflammatory response. Enhancement of PHA response by leptin administration has been documented in two studies of birds (Alonso-Alvarez et al. 2007; Lõhmus et al. 2004) but the generality of those findings awaits for further clarification. On the basis of previous findings we predicted the treatment of leptin to increase immune responsiveness to PHA. The anti-leptin was expected to act as a typical antagonist by binding to leptin receptors with an affinity similar to the non-mutant leptin, and in this way have an opposite effect to the ones observed in leptin treated birds. We also asked whether plasma leptin levels are individually consistent by measuring correlations between individual leptin levels at different stages of the experiment.

Plasma concentrations of leptin were significantly elevated in leptin-supplemented birds after three days of chronic treatment, but did not differ from those of control finches nine days after the surgery. This kind of pattern emerged most likely because of the negative feedback loop that reduced endogenous leptin production. Anti-leptin treatment caused increased plasma leptin concentrations, possibly because the central system reacted to the decrease in leptin signalling by up-regulating the production of endogenous leptin.

Individual plasma leptin levels correlated significantly over six and 21 days (after the effects of treatments had vaned), indicating that at least under some conditions this marker of condition reflects consistent differences between individuals.

A comparison between leptin, control, and anti-leptin treatment groups of greenfinches did not reveal any significant treatment effects on body mass. However, individuals with the greatest increase in plasma leptin concentration lost more weight than birds whose plasma leptin increased less. This observation is in accordance with the 'classic' findings of previous leptin studies in mammals (Ahima and Osei 2004) but contradicts some recent findings in wild birds (Kordonowy et al. 2010; Quillfeldt et al. 2009).

Chronic treatment with leptin significantly decreased the systemic levels of NO_x in greenfinches. This finding is in opposite to the mammalian studies and once more illustrates the difference in leptin function between birds and mammals.

Besides being classically considered as a hormone, leptin is designated as a cytokine as well. In accordance with this, a strong stimulating effect of leptin on T-cell-mediated swelling response was found in old greenfinches. The immune enhancing effect of leptin was observable even when the leptin levels of the leptin-treated group had dropped to the level of saline-treated birds. This provides evidence for a delayed or long-term potentiation of the cells and cytokines involved.

Finding that leptin enhanced swelling responses only in old but not among yearling birds was rather surprising. Similarly, depression of NO production by leptin was stronger in older birds than in yearlings. Very few previous studies have discussed or analysed age effects in leptin physiology, making our findings novel. One possible interpretation of these patterns would be that the interactions between different physiological mechanisms become more sensitive to disturbances with an increasing age, which indeed has been suggested as a mechanism for ageing (e.g., Cichon et al. 2003). Alternatively, yearling birds might present a phenotypically or genetically different cohort of individuals because they have been exposed to selection for a shorter period (e.g., having not been tested for the ability to survive the first winter). Such an explanation would imply very strong natural selection upon the regulation of inflammatory responses by leptin.

In conclusion, this study corroborates that leptin interferes with the nitric oxide system and immune responses. The age dependence of these effects indicates the complexity of underlying regulatory processes and possibly also

strong natural selection on the regulation of inflammatory processes by leptin. Altogether, these findings highlight the value of extending the studies of the functions of leptin from traditional mammal models to passerine birds.

3.2.2. Connections between behaviour and immune responsiveness

Paper IV describes an association between behaviour and immune traits. Animals, including humans, differ systematically in their responses to conspecifics and to their environment and these responses often covary with susceptibility to stress- and immune-related diseases such as cardiovascular disorders, gastric ulceration and various infectious and inflammatory conditions. The ultimate explanation for this covariation is that temporally and/or spatially fluctuating selection pressures favour different stress-coping styles (Bergmüller and Taborsky 2010; Dingemanse et al. 2004) and immune traits (e.g., Lazzaro and Little 2009) under different circumstances. Eventually, life-history trade-offs can also significantly contribute to maintenance of variation in behavioural syndromes (Stamps 2007; Wolf et al. 2007). The proximate mechanism behind such covariation is that neuroendocrine system controls both behaviour and immune responses (reviewed by Koolhaas et al. 1999; Korte et al. 2005).

Despite these conceptual advances, there is very little evidence for a straightforward model of psychological dysfunction, consequent immune disruption, and subsequent disease (Friedman 2008). The progress in the area has been particularly hampered by the use of highly domesticated and often inbred strains of laboratory animals where (perhaps unintended) selection for tameness can efficiently eradicate the whole behavioural phenotypes (Koolhaas et al. 2010). Study of behavioural syndromes in wild animals therefore has a great potential for explaining the ultimate architecture of the connection between health and personality. However, despite the well-established links between behavioural traits and corticosterone responses (e.g., Cockrem 2007), there are no studies on wild bird species associating behavioural traits and immune function directly.

Here I describe such an association in wild-caught captive greenfinches. I take an advantage of variation in behaviour of these birds when brought into captivity: some individuals damage their tail feathers while flapping against cage walls, while others retain their feathers in intact condition for at least three months. This paper asks whether these two bird categories that display different coping with captivity also differ with respect to immune responsiveness. To measure the latter, I applied two assays aimed to describe activation of different components of the immune system, the antibody production against *Brucella abortus* antigen and WBCL response elaborated in Paper II.

The study shows that damage to tail feathers was associated with flapping flight movements and the frequency of such flapping bouts was individually consistent over 57 days. Hence, the condition of tail feathers can be regarded as a behavioural trait, reflecting individuals personality. We found that birds with

intact tails, i.e., relatively "calm" individuals mounted stronger antibody response to *Brucella abortus* (BA) antigen and stronger WBCL response. Notably, the behavioural trait was assessed long before the immune function, i.e., 13 and 19 days before measuring oxidative burst and 25 days before measuring antibody response. Thus, individuals' coping styles with captivity predicted how these individuals would respond to immune challenges in the future. The most parsimonious explanation to this pattern would be association between personality type and immune profile, which is predicted on the basis of the neuroendocrine regulation of both functions (Koolhaas 2008; Korte et al. 2005). This might reflect either stress-induced down regulation of inflammatory and Th1-based responses in birds with damaged tails or permanent differences in the immunoresponsiveness between the birds with different stress-coping styles. To my knowledge, this study provides the first evidence for a direct link between behavioural trait and immunity in a wild bird species.

3.2.3. Carotenoids, immunity and oxidative defences

Paper V investigates the effect of carotenoid supplementation on different immune parameters. Carotenoid-based integument colouration is extremely widespread in animal kingdom. Although animals cannot synthesise carotenoids *de novo*, these pigments are common components of the colour of signals used in mate attraction and other types of social communication. It has been hypothesized that carotenoid colouration is used for communicating the health status of the bearer because carotenoids are efficient immunomodulators and/or antioxidants. However, the latter argument has been recently debated and the mechanisms by which carotenoids modulate immunity or oxidative balance are poorly known.

Here I applied a novel approach for testing whether and how dietary carotenoids affect ROS production by avian blood, using a WBCL assay elaborated in Paper II. We supplemented half of 60 male greenfinches with dietary carotenoids in physiological dose and asked if this affects the parameters of WBCL response. On the basis of published evidence, we had no predictions about the direction of the response. Additionally we measured the strength of humoral immune response on the basis of antibody titres produced against vaccination with *Brucella abortus* antigen. Previous study in another greenfinch subspecies has shown that dietary lutein supplementation increased anti-BA antibody production (Aguilera and Amat 2007); I was thus interested whether we can reproduce this result. I also asked whether induction of inflammatory response *in vivo* depletes plasma carotenoids as demonstrated in some experiments on birds.

The effects of carotenoids on the oxidative burst of phagocytes were tested under neutral conditions and also during *in vivo* immune challenges with *Brucella abortus* (BA) and LPS. Plasma carotenoid levels were successfully increased by 40–53 % in carotenoid supplemented as compared to un-

supplemented birds, but the WBCL response was not affected by carotenoids under any condition. Similarly to the WBCL response, no effect of carotenoid supplementation on anti-BA antibody response was detected, contrary to a study in an another greenfinch subspecies, where lutein-supplemented males mounted significantly stronger anti-BA response than un-supplemented (Aguilera and Amat 2007). vaata, viidetega on siin mingi kala – a ja b sama viite kohta tegelikult, ilmselt mujal ka

Injection of LPS had significant effect on dynamics of individual plasma carotenoid levels between the first and second blood sampling event. During that period plasma carotenoid levels of most birds declined; however this decline was much more prevalent among LPS-injected birds than among saline-injected controls. Results of this study, along with the published evidence thus clearly support the involvement of carotenoids in the inflammatory response.

Altogether these results indicate that interconnections between carotenoid-based colouration, immune function and oxidative stress are much more complicated than originally thought. These findings may be consistent with the view that carotenoids are inefficient antioxidants *in vivo* and, therefore, are unlikely to provide a direct link between oxidative stress and colouration (Hartley and Kennedy 2004; Olsson et al. 2008). Limitation of the current study was that it was not assessed whether the WBCL responses induced in our experiment inflict oxidative damage and whether carotenoids might play role in attenuation of such potential damages. Further studies on these interconnections are thus required before the messages of carotenoid-based animal colouration can be understood.

SUMMARY

When I started this study, it was becoming clear that two rapidly developing disciplines – immunoecology and oxidative stress ecology have a common domain. Production of reactive species for destruction of parasites and pathogens is an integral part of immune defences; however uncontrolled production of these reactive species might lead to collateral damage to the host organisms and cause oxidative stress. Thus it seemed that measuring production of reactive species by immune cells might appear a plausible way for understanding the costs of immune responses, and hence, evolution of optimal immunity. However, immunoecologists lacked suitable tools for this. A single study (Bourgeon et al. 2007) has examined nitric oxide production in an immunoecological context, and when I tried to apply their protocol, I realised that there should be simpler ways for doing this. There were some commercial kits but these were overly expensive, so by combining several previously used methods I developed a simple and user-friendly assay for assessment of NO production from small plasma samples. Similarly, few studies had measured reactive species production by phagocytes in wild animal species but none of these protocols appeared convenient enough. Adjustment of a Pholasin-based whole blood chemiluminescence assay for small samples of avian blood seemed to provide a very suitable tool for measuring production of reactive species by immune cells.

When I started to apply these methods in case studies for solving particular immunoecological questions, I got some negatively and some positively surprising results. For instance, individual changes in coccidian infection intensity between two blood-sampling periods did not predict corresponding changes of NO levels. Contrary to the results obtained in mammals, leptin administration suppressed NO production in greenfinches. Similarly, I predicted that dietary carotenoid supplementation interferes with oxidative burst of phagocytes. That, however, was not the case. On the other hand, the finding that birds with different coping styles with captivity had different WBCL responses (and that this result was reproducible over a weekly interval) was very encouraging, suggesting that this assay has a great potential for the research of interconnections between stress tolerance and immune responsiveness.

Clearly, the methods elaborated and applied in this study do not provide final solutions for major pertinent research problems in immunoecology and oxidative stress ecology. For instance, a clear limitation of my study was inability to assess the potential fitness consequences of reactive species production by immune cells. Captive animals are not particularly suitable for such a purpose, so associations between NO production, oxidative burst and different components of fitness need to be assessed in field studies. Another viable approach would be to assess oxidative stress, e.g., by measuring the extent of oxidative damages to lipids, proteins and DNA. Feasible methods for such measurements are being elaborated in several laboratories and these should

be applied for testing whether and how NO production and WBCL responses inflict oxidative damage. However, such proxies for damage too, need to be tested in the field for associations with components of fitness.

SUMMARY IN ESTONIAN

Oksüdatiivsed kaitsereaktsioonid immuunökoloogilises kontekstis: lämmastikoksiidi produktsiooni ning oksüdatiivse purske mõõtmise meetodite kohandamine ja rakendamine värvulisele

Parasiitide tähtsust evolutsioonilise jõuna on teadvustama hakatud alles viimastel aastakümnetel. Sellest uuest paradigmast on välja kasvanud sellised uued teadussuunad nagu evolutsiooniline parasitoloogia ning immuunökoloogia. Immuunsüsteem kaitseb peremeesorganismi parasiitide ja patogeenide eest, ometi ei ole peremeesorganismide immuunvastused võõrantigeenidele sugugi alati maksimaalsed. Immuunökoloogia uurib immuunvastuste varieeeruvuse põhjusi ja tagajärgi ökoloogilises kontekstis. Kui immuunsüsteem ei suuda organismi ründavate parasiitidega adekvaatselt hakkama saada ja/või kahjustab immuunvastuse käigus iseennast on tagajärjeks haigestumine. Mis takistab immuunsüsteemi paremini toimimast? Ühest küljest on süüdi parasiidid, kes tänu oma lühemale elutsüklile evolutsioneeruvad peremeestest kiiremini. Teisest küljest põhjustab probleeme immuunsüsteemi regulatsioonimehhanismide keerukus ja hävitusmehhanismide ohtlikkus, mis muudab immuunsüsteemi kasutamise kulukaks. Kui organismi mingi funktsioon on kulukas, siis peab see paratamatult teiste funktsioonidega ühise ressursi pärast konkureerima. Nii saaks ka immuunvastusesse paigutatavat ressurssi investeerida hoopis teistesse kohasuse komponentidesse – näiteks signaaltunnuste välja-arendamiseks, sigimiseks või eluea pikendamiseks. Seega eeldavad immuunökoloogid (lähtuvalt elukäikude evolutsiooni teooriale omasest optimaalsusparadigmast), et nii isendi, populatsiooni kui ka kõrgemate taksonite tasandil eksisteerivad lõivsuhted immuunfunktsiooni ning sigimisse, enesesäilitamisse ja signaaltunnustesse tehtud investeeringute vahel. Immuunökoloogia lähenemisviisi aluseks on parasitoloogia, immunoloogia ning evolutsioonilise ökoloogia meetodite rakendamine selle lõivsuhete võrgustiku toimimise kirjeldamiseks.

Algse arvamuse kohaselt peeti põhilisteks immuunvastusega seotud kuludeks energeetilisi, kuid alternatiivse hüpoteesina on pakutud ka immuunvastuses kasutatavate vabade radikaalide jt reaktiivsete osakeste poolt põhjustatud koekahjustusi. Vabad radikaalid (VR) on vähemalt ühe paardumata elektroniga molekulid v. nende fragmendid, mis tekivad mh. hingamisahelas ning osalevad immuunoregulatsioonis ning immuunvastusega kaasnevates fagotsütoosiprotsessides. Vähestes, reguleeritavates ja kontrollitavates kogustes on VR raku elutegevuseks hädavajalikud, kuid ülemäärane ja kontrollimatu VR moodustumine loob eelduse sügavaks oksüdatiivseks stressiks, mille tagajärjeks on biomolekulide (nukleiinhapped, lipiidid, valgud) oksüdatiivsed kahjustused. Nimetatud kahjustuste suhtes on eriti tundlikud neurogenees, immuunoregulatsioon ja MHC molekulide ekspressioon, samuti on oksüdatiivsel stressil võtmeroll mitmetes vananemisega kaasnevates protsessides. Organism saab ennast kaitsta VR reaktsioonide kahjulike tagajärgede vastu antioksüdantide abil, mida võib jaotada eksogeenseteks (nt. E- ja A-vitamiin, karotenoidid) ja

organismi poolt sünteesitavateks e. endogeenseteks (nt. glutatioon, kusihape, albumiin ja antioksüdant-ensüümid).

Immuunvastusega seotud kulude mõõtmine on keeruline, kuna immuunsüsteemiga seotud füsioloogilisi protsessse on palju ning need on vastastikku integreeritud. Raske on ka eristada kontrollitud ning kontrollimatuid protsesse ning samuti on ebaselge, millise tugevusega immuunvastus on optimaalne. Sarnased probleemid ilmnevad ka vabade radikaalide poolt põhjustatud oksüdatiivse stressi mõõtmisel. Ökoloogilisi uurimusi komplitseerivad veel mitmete meetodite liigispetsiifilisus, proovide väikesed mahud, katseloomade surmamise vältimine ja sageli ühekordne proovide võtmise võimalus. Immuunökoloogia areng sõltub seetõttu üsna paljuski uute meetodite väljatöötamisest ja kohandamisest. Käesoleva töö eesmärgiks oligi kahe uue immuunsüsteemi poolt toodetud vabu radikaale mõõtva meetodi kohandamine ja valideerimine ning rakendamine rohevintidel immuunökoloogilistes uurimustes.

Esimene kohandatud meetod (artikkel 1) mõõdab lämmastikoksiidi (NO) tootmist nitritite ja nitraatide kontsentratsiooni kaudu vereplasmas. Lämmastikoksiid on multifunktsionaalne signaalmolekul, mida kasutatakse kehasiseselt paljudeks erinevateks ülesanneteks. Immuunvastuse käigus toodavad makrofaagid lämmastikoksiidi patogeenide hävitamiseks, samas võib ülemäärane NO tootmine põhjustada mitmesuguseid koekahjustusi.

Meetod põhineb Griessi reaktsioonil ning seetõttu tuleb esmalt nitraadid aktiveeritud kaadmiumi terakeste abil redutseerida nitrititeks. Antud meetodi puhul kasutatav keemiline valgu sadestamine on võrreldes alternatiivse ultrafiltratsiooni meetodiga märksa odavam ja kiirem, samas lahjendab see proovi. Kaadmiumil põhinev nitraatide redutseerimine annab võrreldes ensümaatilise redutseerimisega suurema lineaarse mõõtmisvahemiku. Rohevintidel NO tootmist mõõtes selgus, et see üsnagi kiirelt muutuv näitaja on kasutatav immuunvastuse mõõtmisel ja võib anda uudset informatsiooni isendite seisundist. Meetod on suhteliselt lihtne, odav ning vajaminevad proovikogused on väikesed ja seega sobib see hästi ökoloogilistes uurimustes kasutamiseks.

Teine lindudel kasutamiseks kohandatud meetod (artikkel 2) mõõdab kaasasündinud immuunsüsteemi ühte toimemehhanismi – fagotsüütide oksüdatiivset purset. Mõõtmine toimub *in vitro* täisveres ning oksüdatiivse purske esilekutsumiseks kasutatakse bakterite pinna antigeeni lipopolüsahhariidi (LPS). Vastusena LPSi lisamisele täisverele aktiveerivad fagotsüüdid oma NADPH oksügenaasi, mis hakkab tootma vabu radikaale patogeenide hävitamiseks. Antud reaktsiooni saab mõõta vabade radikaalide toimel kiirelt kemiluminesteeruva valgu Pholasini abil. Reaktsioon on oluliselt kiirem ning võimsam kui seni oksüdatiivse purske iseloomustamiseks kasutatud luminestsentsi stimulaatorite puhul, kuna Pholasin võimaldab mõõta sekundite vältel toimuvat ekstratsellulaarset reaktiivsete osakeste produktsiooni, mis toimub vastusena LPSiga stimuleerimisele. Reaktsiooni ulatuslik inhibeerimine superoksiidi dismutaasi poolt viitab, et (vähemalt) peamiselt mõõdeti superoksiidi radikaali vabenemist. Lisaks LPS kasutamisele stimulandina oli teiseks peamiseks meetodi arendamisel tehtud uuenduseks järjestikune stimulandi

lisamine proovile kaheminutilise intervalliga. Niiviisi osutus võimalikus mõõta fagotsüütide aktiveerumise võimet esmase *in vitro* stimulatsiooni järel – enamasti (kuid mitte alati) oli teisene oksüdatiivne purse võimsam kui esimene. Oksüdatiivne purse suurenes vastusena lindude *in vivo* stimuleerimisele erinevate antigeenidega ning sõltus ka lindude bioloogilistest erinevustest, näiteks vanusest.

Järgnevas artiklis (artikkel 3) kasutasin esimeses artiklis välja töötatud NO mõõtmise metoodikat uurimaks rasvarakkude poolt toodetava peptiidhormooni leptiini ning selle antagonisti mõju fütohemaglutiniini poolt tekitatud immuunvastuse tugevusele ning NO tootmisele. Leptiini produktsioon sõltub rasvarakkude hulgast ning seega kannab leptiin infot organismi energeetilise seisukorra kohta. Leptiini kõrge tase mõjub enamasti söögiisu alandavalt ning füüsilist aktiivsust tõstvalt ning madal tase mõjub vastupidiselt. Peale selle on näidatud leptiini taseme tõusu nakkuste ja põletike puhul, mis viitab selle hormooni osalusele immuunvastuses.

Vastupidiselt ennustatule kehamassi muutused menetlusgruppide vahel ei erinenud, kuid sellegipoolest (vastavalt ennustatule) kaotasid kõige rohkem massi linnud, kellel leptiini tase tõusis kõige rohkem. Erinevalt imetajatest alandas rohevintidel leptiini manustamine NO tootmist, mis viitab leptiini erinevatele rollidele lindudel ning imetajatel. Leptiini peetakse ka tsütokiiniks ja sellele vastavalt võimendas leptiini menetlus fütohemaglutiniini poolt esile kutsutud paistetusreaktsiooni, kuid ainult vanadel lindudel. Samuti alandas leptiin NO tootmist kõige rohkem vanadel lindudel. Selliseid vanuselisi erinevusi leptiini funktsioonis pole varem uuritud ja need rõhutavad veelgi selle hormooni keerulist regulatsiooni.

Järgnevalt (artikkel 4) kasutasin teises artiklis välja töötatud metoodikat isiksuseomaduste ja immuunfunktsiooni vaheliste seoste uurimiseks. Selliste seoste uurimisel on osutunud peamiseks takistuseks kodustatud ja laboriloomade kasutamine, sest kodustamise käigus on isendite vaheline käitumuslik varieeruvus oluliselt vähenenud. Isiksuseomaduste ning immunsüsteemi seosed on tulenenud ilmselt muutuvast valikusurvest, mis erinevatel aegadel/kohtades soosib erinevaid stressiga hakkama saamise viise. Vangistusse toodud rohevindid kohanevad erinevalt; mõned neist raplevad vastu puuriseinu ning kulutavad selle käigus oma sabasulgi, samas kui teistel lindudel sellist käitumist ei esine ning saba jääb täiesti terveks. Nende erinevate vangistus-stressiga kohanemise viisidega kaasnesid ka erinevad immuunprofiilid – kulutamata sabaga nn. rahulikel lindudel oli nii kõrgem antikehade vastus uudsele antigeenile kui ka tugevam oksüdatiivne purse (artikkel 2). Huvitav on antud juhul see, et käitumuslikud iseärasused mõõdeti enne immunvastuseid ja seega ennustasid need ette nii omandatud (antikehad) kui ka kaasasündinud (oksüdatiivne purse) immuunsüsteemi osade toimimist. See uurimus on esimene, mis näitab otsest seost isiksuseomaduse ning immunsuse vahel vabalt elaval linnuliigil.

Viimases uurimustöös (artikkel 5) on vaatluse all karotenoidide mõju erinevatele oksüdatiive purske parameetritele ja humoraalsele immuunvastusele uudsele antigeenile. Karotenoidse värvusega katted on loomariigis äärmiselt

levinud. Loomad ei suuda ise karotenoide sünteesida ning saavad neid toidust; sellele vaatamata kasutatakse neid pigmente ohtralt värvussignaalides vastassugupoole meelitamiseks ning muus kommunikatsioonis. Arvatakse, et loomad saavad karotenoidide abil signaliseerida oma tervislikku seisundit, kuna karotenoidid on ka immunostimulaatorid ja/või olulised antioksüdandid. Samas on seda hüpoteesi ka tugevasti kritiseeritud ning mehhanismid, mille abil karotenoidid mõjutaksid immuunsüsteemi või oleksid antioksüdandid, on ebapiisavalt uuritud.

Karotenoidide lisatoitmine ei muutnud rohevintidel oksüdatiivse purske (artikkel 2) käigus toodetavate vabade radikaalide hulka ei neutraalsetes oludes ega ka juhul, kui lindudel oli eelnevalt indutseeritud immuunvastus. Samuti, erinevalt ühest eelnevast samal liigil läbi viidud tööst, ei mõjutanud karotenoidid antikehade vastust uudsele antigeenile. Samas vähendas LPSi süstimine veres tsirkuleerivate karotenoidide kontsentratsiooni, mis viitab karotenoidide osalemisele põletikuprotsessides. Antud tulemused näitavad, et karotenoidse värvuse, immuunsüsteemi ning oksüdatiivse stressi vahelised seosed on keerukamad, kui algselt arvatud. Lisaks toetavad need tulemused arvamust, et karotenoidid ei ole organismis tõhusad antioksüdandid ja seetõttu ei pruugi esineda otsest seost oksüdatiivse stressi ja karotinoidse värvuse vahel.

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PUBLICATIONS

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2005	Tartu University, Institute of Ecology and Earth Science, technician,
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II. Research history

1. Research interests: Immunoecology, oxidative stress, antioxidants

2. Publications

Hõrak, P., E. Sild, U. Soomets, T. Sepp, and K. Kilk. (2010). "Oxidative stress and information content of black and yellow plumage coloration: an experiment with greenfinches." *J Exp Biol* **213**(13): 2225–2233.

Lõhmus, M., E. Sild, P. Hõrak, and M. Björklund. (2011). "Effects of chronic leptin administration on nitric oxide production and immune responsiveness of greenfinches." *Comparative Biochemistry and Physiology – A Molecular and Integrative Physiology* **158**: 560–565.

Renge, I. and E. Sild (2011). "Absorption shifts in carotenoids – Influence of index of refraction and submolecular electric fields." *Journal of Photochemistry and Photobiology A: Chemistry* **218**(1): 156–161.

Sepp, T., U. Karu, E. Sild, M. Männiste, and P. Hõrak. (2011). "Effects of carotenoids, immune activation and immune suppression on the intensity of chronic coccidiosis in greenfinches." *Experimental Parasitology* **127**: 651–657.

Sepp, T., E. Sild, and P. Hõrak. (2010). "Hematological Condition Indexes in Greenfinches: Effects of Captivity and Diurnal Variation." *Physiological and Biochemical Zoology* **83**(2): 276–282.

Sild, E. and P. Hõrak (2009). "Nitric oxide production: an easily measurable condition index for vertebrates." *Behavioral Ecology and Sociobiology* **63**(6): 959–966.

Sild, E. and P. Hõrak (2010). "Assessment of oxidative burst in avian whole blood samples: validation and application of a chemiluminescence method based on Pholasin." *Behavioral Ecology and Sociobiology* **64**: 2065–2076.

Sild, E., T. Sepp, and P. Hõrak. (2011). "Behavioural trait covaries with immune responsiveness in a wild passerine." *Brain Behavior and Immunity* **25**: 1349–1354.

Sild, E.; Sepp, T.; Männiste, M.; Hõrak, P. (2011). "Carotenoid intake does not affect immune-stimulated oxidative burst in greenfinches." *The Journal of Experimental Biology:* **214**: 3467–3473.

3. Conference theses

E. Sild, P. Hõrak, L. Saks, U. Karu, V. Tilgar, P. Kilgas, R. Mänd, poster presentation: "Nitric oxide production in small passerine birds: what does it indicate?" International Society for Behavioral Ecology conference (ISBE 2008) 9–15 august, 2008, Cornell University, Ithaca, New York.

E. Sild, P. Hõrak, oral presentation: "Nitric oxide production- a potentially interesting condition index?" 2nd biannual Baltic meeting "Effects on climate change on avian reproductive biology" 8–9 december 2008, Laitse castle.

E. Sild, P. Hõrak, T. Sepp, poster presentation: "Effects of captivity on hematological condition indices in greenfinches" International Ornithological Congress (IOC 2010) 22–28 august 2010, Campos do Jordao, Brazil.

E. Sild, P. Hõrak, oral presentation: "Carotenoids and innate immunity- insights from measuring oxidative burst in the blood of greenfinches" International Society for Behavioral Ecology conference (ISBE 2010) 26 september-1 october, Perth, Australia.

4. Review work

Reviewed manuscripts for journals Functional Ecology and Methods in Ecology and Evolution.

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Praegune töökoht, amet: Tartu Ülikool, Ökoloogia ja Maateaduste Instituut, tehnoloogiaspetsialist ja doktorant

Haridus

Tartu Miina Härma Gümnaasium, põhiharidus (1999), Tartu Hugo Treffneri Gümnaasium, keskharidus (2002), Tartu Ülikool, loodusteaduse bakalaureuse kraad (2005), Tartu Ülikool loodusteaduse magister (zooloogia ja hüdrobioloogia) (2007).

Keelteoskus: eesti, inglise, vene, rootsi

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2005	Tartu Ülikool, Ökoloogia ja Maateaduste Instituut, loomaökoloogia õppetool, laborant,
2006–2007	SA Teaduskeskus Ahhaa, koolitusjuht,
2008–	Tartu Ülikool, loomaökoloogia õppetool, tehnoloogiaspetsialist

II. Teaduslik ja arendustegevus

1. Peamised uurimisvaldkonnad: Immuunökoloogia, oksüdatiivne stress, antioksüdandid

2. Publikatsioonide loetelu

Hõrak, P., E. Sild, U. Soomets, T. Sepp, and K. Kilk. (2010). "Oxidative stress and information content of black and yellow plumage coloration: an experiment with greenfinches." *J Exp Biol* **213**(13): 2225–2233.

Lõhmus, M., E. Sild, P. Hõrak, and M. Björklund. (2011). "Effects of chronic leptin administration on nitric oxide production and immune responsiveness of greenfinches." *Comparative Biochemistry and Physiology – A Molecular and Integrative Physiology* **158**: 560–565.

Renge, I. and E. Sild (2011). “Absorption shifts in carotenoids – Influence of index of refraction and submolecular electric fields.” *Journal of Photochemistry and Photobiology A: Chemistry* **218**(1): 156–161.

Sepp, T., U. Karu, E. Sild, M. Männiste, and P. Hõrak. (2011). “Effects of carotenoids, immune activation and immune suppression on the intensity of chronic coccidiosis in greenfinches.” *Experimental Parasitology* **127**: 651–657.

Sepp, T., E. Sild, and P. Hõrak. (2010). “Hematological Condition Indexes in Greenfinches: Effects of Captivity and Diurnal Variation.” *Physiological and Biochemical Zoology* **83**(2): 276–282.

Sild, E. and P. Hõrak (2009). “Nitric oxide production: an easily measurable condition index for vertebrates.” *Behavioral Ecology and Sociobiology* **63**(6): 959–966.

Sild, E. and P. Hõrak (2010). “Assessment of oxidative burst in avian whole blood samples: validation and application of a chemiluminescence method based on Pholasin.” *Behavioral Ecology and Sociobiology* **64**: 2065–2076.

Sild, E., T. Sepp, and P. Hõrak. (2011). “Behavioural trait covaries with immune responsiveness in a wild passerine.” *Brain Behavior and Immunity* **25**: 1349–1354.

Sild, E.; Sepp, T.; Männiste, M.; Hõrak, P. (2011). “Carotenoid intake does not affect immune- stimulated oxidative burst in greenfinches.” *The Journal of Experimental Biology:* **214**: 3467–3473.

3. Konverentside ettekanded

E. Sild, P. Hõrak, L. Saks, U. Karu, V. Tilgar, P. Kilgas, R. Mänd, stendettekanne: “Nitric oxide production in small passerine birds: what does it indicate?” Rahvusvaheline käitumisökoloogia konverents (ISBE 2008) 9–15 august, 2008, Cornell University, Ithaca, New York.

E. Sild, P. Hõrak, suuline ettekanne: “Nitric oxide production – a potentially interesting condition index?” 2nd biannual Baltic meeting “Effects on climate change on avian reproductive biology” 8–9 detsember 2008, Laitse loss.

E. Sild, P. Hõrak, T. Sepp, stendettekanne: “Effects of captivity on hematological condition indices in greenfinches”Rahvusvaheline ornitoloogiakongress (IOC 2010) 22–28 august 2010, Campos do Jordao, Brasiilia.

E. Sild, P. Hõrak, suuline ettekanne: “Carotenoids and innate immunity- insights from measuring oxidative burst in the blood of greenfinches” Rahvusvaheline käitumisökoloogia konverents (ISBE 2010) 26 september–1 oktoober, Perth, Austraalia.

4. Muu teaduslik tegevus

Olen retseenseerinud teadusartikleid rahvuvahelistele teadusajakirjadele Functional Ecology ja Methods in Ecology and Evolution.

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS

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