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Redox physiology of wild birds: validation and application of techniques for detecting oxidative stress

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Press

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Author´s contribution to the papers

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

1. INTRODUCTION

Energy allocation between reproduction and self-maintenance is a key life history trade-off. Therefore, forces that provoke constant renewal of an organism's tissues are investigated to understand their role in shaping life. As an organism comes across a hazardous substance, it invokes an energy demanding process to either counteract or tolerate it. Ultimately selection favours those organisms that not only learn to effectively detoxify but also find a use for those harmful substances (Bickham & Smolen 1994). One such substance is oxygen. Since the emergence of aerobic energy production some 3 billion years ago (Sessions *et al.* 2009), aerobic organisms have embraced the challenges imposed by respiration. Inevitable by-products of this electron relocation process are molecules containing an unpaired electron (or molecules prone to give one) collectively called reactive species (RS, Halliwell & Gutteridge 2007). These highly reactive molecules, including reactive oxygen (ROS), nitrogen, chlorine and many more species, pose a threat to the integrity of biological structures (Dröge 2002; Santo *et al.* 2016). Therefore, a variety of antioxidant mechanisms have evolved for billions of years to counteract their harmful effects (Benzie 2000). These mechanisms, including enveloping vital structures, synthesizing RS scavengers and promoting repair of damaged biomolecules, have been fine-tuned over countless generations (Benzie 2000; Gutteridge & Halliwell 2010). Meanwhile RS have also proved useful as a weapon against invading pathogens (Nappi & Ottaviani 2000; Dröge 2002) and signalling molecules (Dröge 2002; Isaksson *et al.* 2011), possibly representing a general signal of cellular stress (Finkel & Holbrook 2000). In light of evidence for the multipurpose nature of RS, oxidative stress (OS) is defined as a disturbance in the pro-oxidant/antioxidant balance in favour of the oxidants, leading to a disruption of redox signalling and control, and/or molecular damage (Sies & Jones 2007). The field that searches for the way(s) in which disruption in redox homeostasis affects fitness through shaping sexual selection, reproduction, ageing and survival (Costantini *et al.* 2010) is called oxidative stress ecology. Together with immunoecology – a field that connects immune responses and disease susceptibility with individual fitness consequences (Sheldon & Verhulst 1996; Schulenburg *et al.* 2009) – this field of research has tried to link OS with major life history trade-offs.

1.1 Oxidative stress – jack of all trades

OS has been held responsible for reduced lifespan. Specifically, cell proliferation in general is thought to be affected by OS, which causes telomere shortening (von Zglinicki 2002) and is long believed to accompany higher metabolic rates (i.e the rate of living hypothesis; Bokov et al. 2004). However, the mechanism by which RS production depends on metabolic state is under debate, as increased mitochondrial energy production decreases its ROS production (Speakman & Garratt 2013). Nevertheless, it has been shown that higher growth

rates can translate into either elevated or reduced antioxidant defences and oxidative damage (reviewed in Metcalfe & Alonso-Alvarez 2010). Furthermore, oxidative damage to macromolecules is shown to increase during ageing (Hulbert et al. 2007), leading to the idea that the rate of senescence might be mediated by a balance between OS and membrane susceptibility to it (Galván et al. 2015).

Reproduction might also increase OS. Cells proliferate rapidly in gonads and antioxidants are allocated into the gametes to promote their survival at the expense of bodily antioxidant defences (Costantini *et al.* 2010; Metcalfe & Alonso-Alvarez 2010). In addition, OS is shown to reduce sperm performance and oocyte maturation, leading to deterioration of gamete quality, aggravated over time (Costantini *et al.* 2010; Metcalfe & Alonso-Alvarez 2010). Furthermore, females may provide oxidative shielding to offspring by reducing their OS levels during breeding at the expense of defence during non-reproducing seasons (Blount *et al.* 2015). Both gamete quality and body condition are directly related to the reproductive potential of an individual, enabling it to tie OS with fitness.

The costs of mounting an immune response can be mediated by OS (Dowling & Simmons 2009). The activation of innate immune response involves release of RS by phagocytes. This process, oxidative burst, is used to kill invading pathogens (Nappi & Ottaviani 2000) but can also non-specifically damage any cells, leading to immunopathology (Halliwell & Gutteridge 2007). Inflammation, especially chronic, has been shown to promote increased oxidative damage and lead to antioxidant depletion even outweighing the direct negative cost of pathogen replication (reviewed in Sorci & Faivre 2009). Although, such an effect is estimated to be small in birds (Costantini & Møller 2009), tolerating pathogens can still cost less than eliciting a prolonged immune response (Medzhitov *et al.* 2012).

Conspicuous sexually selected traits like feather colour or song complexity in birds, may signal the oxidative status of an individual (von Schantz *et al.* 1999). While carotenoids, antioxidants *in vitro*, do not seem to have such an effect *in vivo* (Costantini & Møller 2008; Isaksson & Andersson 2008; Simons *et al.* 2012), melanin-based traits, also under sexual selection (Roulin 2015), are more often shown to be influenced by OS (Galván & Alonso-Alvarez 2008; Hõrak *et al.* 2010; Roulin *et al.* 2011; Henschen *et al.* 2015). Melanin coloration may even signal cognitive abilities (Galván & Moller 2011), which are shown to depend on OS in rodents (Fukui *et al.* 2002; Rosa *et al.* 2007). The few studies associating song and OS have resulted in mixed results (Casagrande *et al.* 2014; Costantini *et al.* 2015a), leaving the question open for debate.

As OS is alleged to affect virtually every major life history trait, it is thought of as a major mediator of life history trade-offs (Dowling & Simmons 2009; Monaghan *et al.* 2009; Costantini *et al.* 2010). Unfortunately, the experimental results often fail to conclusively demonstrate such a role for OS (Isaksson *et al.* 2011; Selman *et al.* 2012). Typically, the occurrence of OS is established by detecting changes in few antioxidant or oxidative damage markers (Costantini

2008). Yet, the redox status of an organism is dependent on a delicate balance of pro- and antioxidants, which have various regulatory roles within an individual (Finkel & Holbrook 2000). Unfortunately, only by quantifying all important mediators of the redox state in different tissues and timeframes (Hõrak & Cohen 2010) can the actual role of OS in the abovementioned trade-offs be established.

1.2 Quantifying redox state – decades of perplexity

Advancement in oxidative stress ecology is much hindered by a lack of reliable measurement techniques (McGraw *et al.* 2010). There is great number of methods used to estimate *in vivo* concentrations of various endo- and exogenous antioxidants, RS, and damaged lipids, proteins or DNA (reviewed in Dalle-Donne *et al.* 2006; Halliwell & Gutteridge 2007; Knasmüller *et al.* 2008). However, currently none of the available methods fulfils the required technical criteria (high specificity, full validity, good repeatability and simplicity of measurement) for any given biomarker of OS (Halliwell & Gutteridge 2007). Moreover, the usefulness of many measured biomarkers is doubtful, as some have questionable role *in vivo* (e.g. resistance of plasma components to *in vitro* generated severe oxidative insult (Halliwell & Gutteridge 2007)), depend on diet (e.g. uric acid (Cohen *et al.* 2007), reactive oxygen metabolites (ROMs, Pérez-Rodríguez *et al.* 2015; Butler *et al.* 2016) and lipid peroxidation (Pérez-Rodríguez *et al.* 2015)) or fluctuate in time due to unknown reasons (e.g. total antioxidant capacity and lipid peroxidation (Galván & Alonso-Alvarez 2009; Sepp *et al.* 2012b)). Proper biomarker selection is therefore of utmost importance.

A single measure of OS is insufficient to declare an occurrence of OS (Hõrak & Cohen 2010; Selman *et al.* 2012). There are differences in the removal/repair time of different damaged biomolecules (Halliwell & Whiteman 2004), and different tissues differ in damage susceptibility (Medzhitov *et al.* 2012). Therefore, careful measurement time and tissue selection is essential. Moreover, while selecting biomarker combinations, it is crucial not only to include both pro- and antioxidant markers (Halliwell & Gutteridge 2007; Costantini & Verhulst 2009) but also to select several biomarkers within each category to cover all arms of antioxidant defence, oxidative damage and pro-oxidant generation (Cohen & McGraw 2009; Romero-Haro & Alonso-Alvarez 2014). Unfortunately, ecological studies generally require non-invasive methods to allow longitudinal sampling from a small amount of easily obtainable tissue (Pedersen & Babayan 2011). Consequently, quantifying redox state in wild animals has to rely on a small subset of OS biomarkers measurable from blood or other easily obtainable tissues. Such limitations further complicate the process of linking oxidative stress to life history trade-offs. Only when advances in measurement techniques allow a better overview of the physiological state of an organism can the role of OS in life history trade-offs be properly investigated (Speakman *et al.* 2015).

1.3 Aims of the thesis

This work aims to characterise the usability of some widely used markers of OS and antioxidant capacity in ecological studies. In particular I want to highlight (1) the importance of proper biomarker selection in determining the occurrence of OS in ecological setups and (2) emphasize the general inability to reliably demonstrate disruption in redox homeostasis with currently available biomarkers under ecologically relevant stressors.

There is a lack of consensus on ways of determining oxidative stress in free ranging animals (Costantini & Verhulst 2009; Garratt & Brooks 2012; Selman *et al.* 2012). While OS cannot be accurately assessed using a single marker and is tissue dependent (Dotan *et al.* 2004; Hõrak & Cohen 2010) studies of oxidative stress ecology have mainly relied on biomarkers from easily obtainable tissues like blood (Monaghan *et al.* 2009). I aimed to clarify which of the popular blood-based measures of antioxidant capacity and oxidative damage (to proteins, lipids and DNA) prove to be most useful in detecting mild to severe oxidative insult in wild birds after an experimentally induced oxidative insult in captive greenfinches (*Carduelis chloris*, **Paper I**). Subsequently, I aimed to assess the applicability of d-ROMs assay. This assay has, despite concerns about its selectivity in detecting ROMs (Lindschinger *et al.* 2004; Erel 2005; Harma *et al.* 2006; Lindschinger & Wonisch 2006; Buico *et al.* 2009; Ganini *et al.* 2012), become increasingly popular amongst studies of OS ecology and is actively promoted by some researchers (see Costantini 2016). I aimed to clarify how much of the detected signal can be attributed to changes in redox status in different animal species and whether the assay can be modified to meet its purpose (**Paper II**).

With an idea of usable blood-based biomarkers in place, I looked at OS under naturally occurring situations. As immune system activation is thought to cause OS via enhanced ROS production to combat pathogens (Sorci & Faivre 2009), I subsequently asked if a disruption to redox homeostasis can be detected under immune system activation with bacterial lipopolysaccharide (LPS) injection (**Paper III**). Furthermore, advances in transcriptome profiling enabled an elaboration of the underlying sources of the variation. Changes in redox regulation during acute phase response might stem from changes within the measured tissue as well as originate from others (Speakman & Garratt 2013). I asked how gene expression in blood is adjusted to maintain homeostasis following immune system stimulation via LPS injection (**Paper IV**). Finally, as ageing is supposedly affected by OS (Costantini *et al.* 2010; Nussey *et al.* 2013) and links between biomarkers of OS and longevity are rarely evaluated from blood of non-human vertebrates (Stier *et al.* 2015), I tested for the effect of age on some blood based OS biomarkers in a long lived seabird, the common gull (*Larus canus*, **Paper V**).

2. MATERIALS AND METHODS

2.1 Study systems

2.1.1 Greenfinches

The European greenfinch is a seed-eating passerine bird belonging to the family *Fringillidae* (Zuccon *et al.* 2012). Adult birds weigh around 30 g and have a wing span of 25 cm. Greenfinches produce multiple broods per year with an average clutch size of 4 eggs (Bensouilah *et al.* 2014). Birds are sexually dichromatic and male carotenoid-based plumage coloration is under sexual selection (Eley 1991) and sensitive to infections (Merilä *et al.* 1999; Lindström & Lundström 2000; Hõrak *et al.* 2004). Greenfinches are a good model species for ecophysiological research as they cope well in captive conditions. The species has been previously studied for carotenoid metabolism (Peters *et al.* 2008), immune function (Aguilera & Amat 2007; Sarv & Hõrak 2009), chronic infections (Lindström *et al.* 2001; Sepp *et al.* 2012a), oxidative stress (Hõrak *et al.* 2007; Hõrak *et al.* 2010; Herborn *et al.* 2011b; Sepp *et al.* 2012a), personality and behaviour (Lilliendahl 2000; Herborn *et al.* 2011a; Herborn *et al.* 2011b; Sild *et al.* 2011a; Sepp *et al.* 2014).

Male and female greenfinches that contributed to this thesis were captured with mist-nets in January of 2012 and 2013 at bird feeders in the city of Tartu, Estonia (58°22' N, 26°43' E). The birds were housed indoor in individual cages (27x51x55 cm) with sand-covered floors and released after two months. Male and female birds were housed in separate aviaries, where they had visual contact with their neighbours. The birds were supplied *ad libitum* with sunflower seeds and tap water. The natural day-length cycle was maintained in the aviaries using artificial lighting by luminophore tubes.

2.1.2 Common gulls

The common gull is a monogamous long-lived seabird from the family *Laridae*. Adult birds weigh around 400 g and have a wingspan of 120 cm. The common gull breeds in colonies where it lays a clutch of 3 eggs. Both parents provide parental care. Breeding usually starts at the age of 3 and lasts on average 5–6 years (Rattiste 2004), although it may continue over 30 years. Nevertheless, after ten years of breeding indications of reproductive senescence emerge as breeding success declines (Rattiste 2004). Gulls exhibit sexually dichromatic wing ornamentation reflecting individual quality (Sepp *et. al* in prep.) and displays conspicuous carotenoid based coloration on bill and legs.

Gulls for the current study were caught from years 2008 to 2010 at the islet of Kakrarahu in Matsalu National Park (west coast of Estonia; 58°46' N, 23°26' E) using spring traps. The colony has been continuously monitored since 1962. Hence, its population structure is well documented. According to Rattiste (2004), over half of the males return to their birth colony to breed and less than

3% switch breeding colonies between years. Therefore, such a study system enables the collection of longitudinal individual-based data from first to last breeding attempt.

2.2 Studied biomarkers

This thesis evaluated only blood-based biomarkers of oxidative stress (OS) for their suitability to detect changes in redox physiology. Both blood plasma and cells were used. From numerous methods for quantifying antioxidant status or oxidative damage (reviewed in Dalle-Donne *et al.* 2006; Halliwell & Gutteridge 2007; Knasmüller *et al.* 2008) I measured a subset of OS biomarkers that has been extensively used in studies of avian ecology.

Biomarkers of antioxidant status were measured both from blood plasma and cells. Plasma total antioxidant capacity (TAC) was assessed by measuring Trolox equivalence antioxidant capacity. The method evaluates plasma capacity to scavenge *in vitro* hydrogen peroxide generated radicals (Somogyi *et al.* 2007). In addition, plasma uric acid concentration was determined using a commercial kit relying on plasma reaction with uricase (uric acid liquicolor, HUMAN, Wiesbaden, Germany). Uric acid has important antioxidant capacities and has been shown to significantly contribute to TAC values (Cohen *et al.* 2007). Oxygen radical absorbance test (OXY, Diacron International, Grosseto, Italy) was performed to add another possible measure of total plasma antioxidant status. The test evaluates plasma ability to cope with *in vitro* oxidant action of hypochlorous acid. Total glutathione (GSH) concentration from blood cells was assessed following a method described by Alonso-Alvarez *et al.* (2008). The assay reduces sample glutathione disulfide to GSH, which forms a coloured end product with a chromophore. Lastly, carotenoid concentration, a possible exogenous contributor to plasma antioxidant status, was determined using a simple spectrophotometrical method previously described by Sild *et al.* (2011b).

Biomarkers of oxidative damage to lipids, proteins and DNA were evaluated either from blood plasma or cells. Oxidative damage to DNA of blood cells was assessed using single cell gel electrophoresis, i.e. the comet assay (Collins 2009). Detection of oxidised DNA bases (mainly 8-oxo-7,8-dihydroguanine) was achieved by combining the alkaline version of the assay and sample treatment with bacterial repair endonucleases. Oxidative damage to proteins is reflected by the amount of carbonyl groups in the sample (Requena *et al.* 2003). The total amount of protein carbonyls in blood cells were measured as previously described (Qujeq *et al.* 2005). The assay relies on spectrophotometric detection of coloured adduct that forms after a reaction between 2,4-dinitrophenylhydrazine and sample carbonyls.

The amount of lipid peroxidation was assessed from serum samples. Stable end products of lipid peroxidation include mainly aldehydes (e.g. malondialdehyde, MDA), alkanes or isoprostanes (Mateos & Bravo 2007). This thesis included plasma MDA quantification using liquid chromatography mass spec-

trometry analysis (Andreoli *et al.* 2003). This technique avoids the shortcomings of the classical MDA quantification assay that utilises thiobarbituric acid. A combination of all damaged biomolecules is supposedly detected by the d-ROMs test (Diacron International, Grosseto, Italy). This test aims to measure organic hydroperoxides from serum (Costantini 2016). The assay relies on Fenton reaction that generates peroxyl radicals from sample hydroperoxides. Generated peroxyl radicals react with a chromogen to produce a coloured chromogen radical.

2.3 Ethics of the experiments

Experiments that were carried out in this thesis comply with the current laws of Estonia and were approved by the Estonian Ministry of the Environment (licence no. 1-4.1/11/100, issued on 23 March 2011) and by the Animal Procedures Committee of the Estonian Ministry of Agriculture (decision no. 95, issued on 17 January 2012). These licences granted permission to:

- Catch greenfinches and other bird species not in the list of endangered species.
- Take blood and feather samples in an amount that has been previously amply reported not to be harmful for the species in question.
- Bring the birds into a laboratory for a restricted time for measurements and analyses, releasing them into the capture location in good condition.
- Gather other kinds of necessary data about the reproductive and other life history traits of birds in a restricted scale.
- Generate oxidative stress using non-lethal doses of paraquat
- Treat natural coccidian infection and infecting birds with coccidian strains
- Immune stimulate birds with *E. coli* lipopolysaccharides and *Brucella abortus* dead vaccine

The studies compiled with the organizational conditions of the experiments, which were stated in the licences.

3. RESULTS AND DISCUSSION

3.1 Measuring OS under experimentally generated oxidative insult (I)

Progress in oxidative stress ecology is impeded until issues of what and how to measure are solved. *In vivo* generation of oxidative stress (OS) is particularly needed to clarify links between fitness and antioxidant levels or oxidative damage (Pérez-Rodríguez 2009). In the first paper I addressed this question by administering a pro-oxidant compound, paraquat, through the drinking water of female captive greenfinches. Like diquat, paraquat generates oxidative stress by producing superoxide anions (Dinis-Oliveira *et al.* 2008). It is widely used for the generation of OS in biological systems (Halliwell & Gutteridge 2007; Knasmüller *et al.* 2008) and is probably the most suitable molecule for chemical induction of OS (Koch & Hill 2016). The experiment aimed to test how different levels of experimentally generated OS reflect in blood-based parameters of antioxidant protection and oxidative damage to all major macromolecule classes. Antioxidant parameters under inspection included "total" antioxidant protection markers from plasma (total antioxidant capacity (TAC) and oxygen radical absorbance test (OXY)) and individual antioxidants (erythrocyte glutathione (GSH), plasma uric acid and carotenoids). Oxidative damage was quantified to plasma lipids (malondialdehyde, MDA), erythrocyte proteins (protein carbonyl levels) and DNA.

The experiment succeeded in disrupting the redox homeostasis of paraquat administered birds, as birds from the treatment group receiving 0.2 g/L paraquat lost significantly more body mass, had increased oxidative damage to erythrocyte DNA and showed an increase in erythrocyte GSH levels. Unfortunately this dose also generated 50 % mortality in this treatment group. Surprisingly none of the other measured parameters were affected either by high or low (0.1 g/L) dose paraquat treatment.

The obtained results comply with the few other studies using experimentally induced OS in wild birds (Galvani *et al.* 2000; Isaksson & Andersson 2008; Galván & Alonso-Alvarez 2009). Such results may imply either that **(a)** many of the popular biomarkers of OS are fairly insensitive in detecting OS. Indeed, the few other studies that have used known sources reactive oxygen species (ROS) have failed to detect changes in lipid or protein peroxidation markers or antioxidant markers including TAC and uric acid (Galván & Alonso-Alvarez 2009; Lucas *et al.* 2014). It is also possible that, **(b)** the usage of few antioxidant and damage markers from a single tissue is not enough to draw conclusions about the occurrence of OS in an animal as a whole. Encompassing measurements from different tissues has shown that changes in oxidative damage or antioxidant concentration can be detected in some while absent in other tissues depending on the manipulation conducted (Galvani *et al.* 2000; Kammer *et al.* 2011; Guerra *et al.* 2012; Hermes-Lima *et al.* 2012; Marasco *et al.* 2013). Such a discrepancy between tissues may result from differences in susceptibility to

oxidative insult and tissue regeneration times (Speakman *et al.* 2015). Another possibility is that **(c)** disruption in redox homeostasis is naturally occurring only under severe pathophysiologies. In humans OS can be detected only with some severe diseases (Halliwell & Gutteridge 2007), so it is likely that under mild increases of ROS bodily antioxidant mechanisms or reserves are sufficient to prevent OS (Lucas *et al.* 2014). The organism's ability to successfully counteract increases in ROS seems especially likely considering the number of generations organisms have lived in environments rich in oxygen (Benzie 2000). In any case (**a**, **b**, **c,** or a combination thereof) the current experiment implies that the chances of detecting OS from those blood-based measures are poor in ecological studies of animals under naturally occurring stressors. Especially considering that the response to oxidative stimulus may be time lagged and different parts of the redox balance machinery have different response times (Khassaf *et al.* 2001; Vider *et al.* 2001; Pedraza-Chaverrí *et al.* 2005). In such a situation only the response to elevated ROS may be detectable. Specifically, from the measured markers only blood GSH levels and DNA damage may have diagnostic value, as these biomarkers respond to environmental stimuli more often than others (Isaksson 2010; Keles *et al.* 2010; Rodriguez-Estival *et al.* 2010; Freeman-Gallant *et al.* 2011). However I managed to show changes in GSH and DNA damage only under severe OS, which is not likely to accompany common life history events. Therefore this study further highlights methodological hurdles in detecting OS in wild animals.

3.2 d-ROMs assay is not suitable for detecting OS (II)

In recent years, despite its lack of specificity in detecting oxidative metabolites, the d-ROMs test has become the most commonly used method to measure OS in wild animals (Costantini & Dell'Omo 2006; Bonisoli-Alquati *et al.* 2010; Costantini & Bonadonna 2010; van de Crommenacker *et al.* 2010; Casagrande *et al.* 2012; Isaksson 2013; Schneeberger *et al.* 2013). Any attempts to criticise the selectivity of d-ROMs assay towards measuring ROMs (Lindschinger *et al.* 2004; Erel 2005; Harma *et al.* 2006; Lindschinger & Wonisch 2006; Buico *et al.* 2009; Ganini *et al.* 2012) have faced fierce accusations of methodological inaccuracy (Iorio & Balestrieri 2005; Banfi *et al.* 2006; Costantini 2016). Furthermore, results providing evidence for the shortcomings are sometimes interpreted as supportive (Colombini *et al.* 2015; Abuelo *et al.* 2016; Butler *et al.* 2016). I aimed to disentangle whether the assay could be modified to selectively measure serum ROMs. For that purpose d-ROMs kit readings from human and avian serum as well as commercial ceruloplasmin (CP) and H_2O_2 solutions were compared under different assay conditions. The paper examined the effects of temperature and the availability of iron or endogenous antioxidants (uric acid, GSH, albumin) to the d-ROMs assay readouts. In addition, serum d-ROMs values were compared with the serum ferroxidase activity of two wild bird species from the genus *Carduelis*.

The results showed clearly that serum d-ROMs test values of wild birds correlate strongly with serum ferroxidase activity and are more temperature sensitive than could be expected from simple Fenton reaction. In addition the assay readouts depended on serum iron availability and albumin content. Uric acid did not affect the assay readouts.

As expected, a correlation between serum ferroxidase activity and d-ROMs values was found in wild birds, consistent with previous results in humans (Erel 2005). However, the absolute values for both measures turned out to be much lower in avian species compared with measurements from the tested mammalian species. This implies that the serum components producing the signal have either a much lower concentration or are functionally different in birds. Furthermore, the temperature dependence kinetics of the d-ROMs assay suggested enzymatic contribution to the assay readouts. Although CP ferroxidase activity can be blocked with azide, its concentrations required for sufficient inhibition also affect the detection of ROMs. In addition, iron availability as well as other serum metals (Abuelo *et al.* 2016), contribute to assay readouts. Altogether our results suggest that the d-ROMs assay cannot be adjusted to accurately quantify serum reactive oxygen metabolites (ROM) content, as too many physiologically variable serum components contribute to the signal. Therefore, many of the experiments declaring detection of OS based on d-ROMs test (e.g. Bonisoli-Alquati *et al.* 2010; Costantini & Bonadonna 2010; Costantini *et al.* 2015b), especially those that have linked OS and immune responses (e.g Costantini & Dell'Omo 2006; van de Crommenacker *et al.* 2010; Casagrande *et al.* 2012; Schneeberger *et al.* 2013) would need re-evaluation. Often these results can be easily explained through changes in inflammatory responses as many well known positive acute phase proteins like CP (Georgieva *et al.* 2010) or ferritin (Lawson *et al.* 1989) display ferroxidase activity and are also known to respond to various non-inflammatory conditions (Murata *et al.* 2004). Indeed, many conditions, including inflammation, may cause OS but the d-ROMs test is not suitable to selectively distinguish the underlying cause of an observed change. To add another level of confusion, even the promoters of the d-ROMs assay cannot decide whether it estimates production of free radicals, oxidative damage or ROMs (Costantini 2016). Hence, a change in the d-ROMs assay readouts does not necessarily mean a change in OS levels, an interpretation too often used in studies of oxidative stress ecology.

3.3 The effect of immune challenge on biomarkers of OS (III)

Fending off parasites and pathogens is presumably costly (Sheldon & Verhulst 1996). These costs may be energetic (Klasing 2004), immunopathological (Graham *et al.* 2005) or stem from trade-offs within the immune system (Ardia *et al.* 2011). In any case such costs may be mediated by OS (Dowling & Simmons 2009). I aimed to find out whether experimentally inducing different types of immune response (via injection of lipopolysaccharide LPS and

Brucella abortus (BA) antigen) or psychologically stressing the birds (via exposure to a predator image) results in OS. To include the costs of possible interactions between LPS and psychological stress a 2*2 factorial experiment was conducted. The redox state of birds was assessed measuring changes in antioxidant (erythrocyte GSH, uric acid, TAC, OXY) and oxidative damage (erythrocyte DNA damage and protein carbonyls) markers. In addition I looked at the differences in overall body condition and BA antibody concentration. The timing of blood sampling was chosen so that it would be comparable to the chemical induction of OS in **paper I**.

The results showed that although the LPS treatment appeared to be costly in terms of reduction in body mass, similarly to chemical induction of OS (**Paper I**) only an upregulation in antioxidant defences (measured as increased GSH and OXY) was detected among the LPS injected birds. However, change in OXY correlated with change in total plasma proteins: ($r=0.53$, $p=0.01$, $n=23$). None of the other biomarkers tested reacted significantly to LPS injection. BA injection and psychological stress failed to cause detectable changes in any of the measured parameters.

Indeed, according to a meta-analysis by Isaksson (2010) GSH appears to be the most sensitive antioxidant marker. On the other hand, LPS-induced immune challenge (Jaeschke 1992; Portolés et al. 1996) or higher parasite prevalence (Lopez-Arrabe et al. 2015) generally result in reduced GSH levels. Undeniably, the timing of measurements may be a key factor causing such discrepancies (see Fig. 2 in Paper I). Likewise, immunostimulation has been shown to decrease plasma OXY values when measured up to 24h (Costantini & Dell'Omo 2006; van de Crommenacker et al. 2010), increase if measured 2-3 days (this study and Marri & Richner 2015) and again decrease if measured 6 days (Casagrande et al. 2012) post injection. However confirmation of such patter would require a time course study. Yet observing changes in OXY or GSH do not necessarily mean an occurrence of OS. GSH has various roles within an organism from regulating cell proliferation and apoptosis to cytokine production and detoxification of xenobiotics (reviewed in Wu et al. 2004). Likewise, measuring the resistance of a biological matrix to high concentrations of HClO in vitro (OXY test), is not indicative of its antioxidant capacities under in vivo occurring oxidative insults (Halliwell & Gutteridge 2007). Furthermore, changes in OXY may just indicate a change in total plasma proteins, which this study clearly demonstrates (change in these two biomarkers were strongly correlated). The finding that no other measure of oxidative damage or antioxidant status responded to the LPS treatment further undermines the possibility to interpret the observed change as an alteration in redox homeostasis. In chickens and white-browed sparrow weavers (Plocepasser mahali), immune stimulation had no effect on plasma TAC or uric acid levels (Cohen et al. 2007; Cram et al. 2015), similarly to this study. Still, many earlier studies have claimed that immune stimulation affects markers of OS (summarized in Costantini & Møller 2009), so the role of OS in mediating immune system related trade-offs remains elusive.

Psychological stress had no effect on any of the measured parameters. Although the applied stressor influenced locomotor activity of the birds (Männiste *et al.* 2013), no differences in feather corticosterone concentrations were observed. It is possible that mild acute stress (detectable changes in behaviour up to 2 days) does not affect antibody production or redox homeostasis. However, if the applied stressor could be considered ecologically relevant, occurrence of OS in wild animals under such behavioural stressors would be extremely hard to detect. Clearly, distinguishing actual occurrence of OS from inflammation or other processes that alter body condition is hard, especially if only a few currently popular blood-based biomarkers of OS are used. Therefore, a more complete picture of changes during an immune response should be obtained.

3.4 Immune challenge induced transcriptional changes in avian blood (IV)

Physiological processes underlying immune function are more complex than acknowledged (Schmid-Hempel 2005; Pedersen & Babayan 2011). Hence, research in immunoecology would benefit greatly from a more complete picture of physiological processes following an immune stimulation. Quantifying transcriptional changes in blood, the preferred sampling tissue for ecological studies (Monaghan *et al.* 2009; Pedersen & Babayan 2011), may thus offer a complimentary way to understand the underlying processes in the tissue. In order to further investigate the response to experimentally induced immune challenge, I injected 8 female greenfinches either with LPS or saline and subsequently (12h later) quantified the full transcriptional profile of their blood cells.

Altogether 66 084 different RNA sequences were identified from the whole blood transcriptome. From those sequences 86% s mapped to the zebra-finch (*Taeniopygia guttata*) genome. However only ~44% of these found a match from Uniprot-SwissProt database and again only around half of the latter were unique genes. In total immune stimulation significantly changed the expression of 1911 transcripts from which 466 were annotated.

As a large part of the mammalian transcriptome is not characterised (McGettigan 2013) and even genes absolutely essential for a fully functional organism often lack annotations (Hutchison *et al.* 2016), the obtained annotation efficiency of greenfinch transcriptome could be considered satisfactory. Especially as most of the sequences mapped to zebra-finch genome and all of the conserved core eukaryotic genes (Parra *et al.* 2007) were present in the dataset.

Immune challenge clearly influenced the gene expression pattern of blood cells. However, only a quarter of the up- or downregulated transcripts was functionally annotated, so most of the differentially regulated genes had unknown function. Of all of the differentially regulated transcripts most of the annotated genes pointed to an induction of cellular rearrangement, specifically,

enhanced protein catabolism (ubiquitin mediated proteolysis) was confirmed. Such a pattern coincides well with recent study on red-legged partridges (*Alectoris rufa*) (Sevane *et al.* 2015). Most of strongly upregulated genes, including gallinacin-2, avidin (AVID), serum amyloid A and protein MRP-126, are known acute phase proteins (Tuohimaa *et al.* 1989; Uhlar & Whitehead 1999; Figdor *et al.* 2002; Matulova *et al.* 2012; Cuperus *et al.* 2013), suggesting an on-going acute phase response. Upregulation of AVID following immune challenge has also been shown in skin, cecum and spleen samples of chicken and red-legged partridges (Matulova *et al.* 2012; Matulova *et al.* 2013; Sevane *et al.* 2015), rendering AVID the most useful transcript for robust detection of acute phase response in avian species.

Disruption of redox homeostasis in this study was hinted at by the upregulation of some subunits of DNA repair complex TFIIH and some promoters of antioxidant protection (copper-transporting ATPase 1, sirtuin, Superoxide dismutase 1 and ferritin). However many other OS related genes present in the dataset were either downregulated (glutathione peroxidase 1, 70kDa heat shock proteins) or remained unaltered (e.g. several peroxiredoxins, thioredoxins, glutathione S-transferases). Furthermore, no OS related pathway emerged from the available annotated transcripts, similarly to transcriptome profiling of immune stimulated red-legged partridges (Sevane *et al.* 2015). Hence, disruption of redox homeostasis in blood cells following induction of innate immune response remains rather hypothetical based on our current knowledge of gene functions.

3.5 Ageing and markers of OS (V)

Accumulating evidence from different taxa suggests that life-history traits associated with reproduction and survival depend on age (reviewed in Nussey *et al.* 2013; Fletcher & Selman 2015). However, the physiological mechanisms explaining age-related declines in such traits are poorly understood. Senescence has been proposed to result from OS either by direct damaging of biomolecules by ROS (Harman 1956; Kirkwood & Kowald 2012) or by a balance between disruption in redox homeostasis and membrane fatty acids susceptibility to ROS attack (Galván *et al.* 2015). Although many studies have looked at how OS relates to senescence, they have mostly been done on short-lived laboratory model organisms (Costantini *et al.* 2010). Only recently have natural populations been included (Galván *et al.* 2012; Bize *et al.* 2014; Herborn *et al.* 2015; Rattiste *et al.* 2015). Still, rarely these studies include more than a few markers of OS. I aimed to test whether several markers of antioxidant protection or oxidative damage correlate with age or predict lifespan in a long-lived seabird, the common gull. Antioxidants measured over multiple years included erythrocyte GSH, uric acid, TAC and carotenoids. From oxidative damage markers, lipid peroxidation was measured. In addition, overall body condition was assessed by quantifying plasma protein and triglyceride concentrations.

The obtained results did not indicate age related increase or decrease in any of the measured OS parameters. However female gulls with lower GSH levels tended to live longer, while none of the other OS biomarkers predicted survival. Nevertheless, age related senescence in reproductive functions was evident among the birds.

Although the study confirmed previously shown age related decline in breeding success (Rattiste 2004; Brommer *et al.* 2009), no age related decline in the measured parameters of OS were observed. Indeed, more and more data refute a simple link between OS and ageing (reviewed in Speakman & Selman 2011; Speakman & Garratt 2013; Rattiste *et al.* 2015). It is possible that some functions of an organism deteriorate faster than others (Nussey *et al.* 2013), so disruption in redox balance may not coincide spatially or temporally with reduction in reproductive performance. More often than not OS parameters in different tissues do not correlate with each other (Speakman *et al.* 2015) and show different temporal patterns of up- or downregulation (Khassaf *et al.* 2001; Vider *et al.* 2001; Pedraza-Chaverrí *et al.* 2005). The only antioxidant marker associating with lifespan in this study was total erythrocyte GSH. As elevated glutathione levels may suggest occurrence of past oxidative insult via compensatory up-regulation of antioxidant defences (Paper I and Trzeciak *et al.* 2012), these results may perhaps indicate that OS impacts the lifespan of long-lived birds. Yet, by no means the bulk of OS markers measured in this study can be considered comprehensive. Furthermore I discourage drawing conclusions from a single measure of OS from a single tissue, as many markers of OS measure a variety of processes (Halliwell & Gutteridge 2007; Paper II). Thus, it would be unwarranted to declare that OS shapes ageing in natural populations, although current knowledge is also insufficient to refute the idea.

CONCLUSIONS

Identifying proper biomarkers for measuring occurrence of oxidative stress (OS) in ecological setups highlighted the usefulness of measuring erythrocyte glutathione (GSH) and deceptiveness of d-ROMs assay. From all of the biomarkers of OS that had their validity assessed in this thesis, only one marker of antioxidant protection, erythrocyte GSH concentration, detectably reacted to chemical induction of OS (**Paper I**), was influenced by immune stimulation (**Paper III**) and was associated with the lifespan of birds (**Paper V**). However, sensitivity to natural stressors and OS does not necessarily mean that changes in GSH concentration are undeniably indicative of OS. Reliably capturing the redox state of an individual requires both excellent knowledge of its general physiology and current health state (Cram *et al.* 2015). Importantly, many markers used to measure OS also quantify the state of various components within an organism. This thesis highlights the confounding factors influencing the readings from an increasingly popular d-ROMs assay and discourages its use in studies of oxidative stress ecology (**Paper II**). Using only few biomarkers of antioxidant status and oxidative damage is highly error prone and thus should be avoided in studying the constraints posed by reactive oxygen species ROS production, as the exact number of tissues, measurement timepoints and markers to measure, is context dependent (Dotan *et al.* 2004).

It remained unclear to what extent OS mediates life-history trade-offs under naturally occurring stressors. While psychological stress failed to induce disruption of redox homeostasis (**Paper III**), induction of immune response failed to clearly result in OS (**Paper III**), even if the whole transcriptomic profile of bird blood was quantified (**Paper IV**). Clearly, sampling only one tissue is not enough to state anything about the actual occurrence of OS in the whole animal. Unfortunately, currently available techniques of non-terminal sampling limit the possibilities of using tissues other than blood (Speakman *et al.* 2015). Therefore most studies on OS in natural populations rely on measurements taken from easily obtainable tissues. However, too often such studies of OS rely on the premise that more antioxidants AO and/or less RS are beneficial for an organism (Costantini & Verhulst 2009), while the multidimensionality of their roles calls for a delicate balance of those substances (Dröge 2002). This balance is shaped by natural selection to fit the needs of an organism (Gutteridge & Halliwell 2010). It is possible that elevated ROS levels are so well counteracted within the body that the only observable cost is energetic (reduction in body mass in papers **I**, **III** and **IV**). Indeed, even during reproductive senescence, birds seem to maintain their redox balance in blood, so that OS cannot be observed during ageing (**Paper V**), adding further support to the need to revisit the free-radical damage theory of ageing (Speakman & Selman 2011). However, as dealing with oxygen toxicity is so well integrated into all aerobic animals, finding out if ROS production is actually constraining life history strategies (i.e. driving the evolution of optimal investment patterns) requires a far more complex approach than employed hitherto.

SUMMARY

Oxygen is a noxious molecule that easily forms radicals that readily react with biological matrices. Hence, in the course of evolution organisms have developed various types of antioxidant defence in order to protect themselves against toxic effects of O_2 while harvesting its power in energy production. For a long time oxidative stress, a body-damaging imbalance between reactive oxygen species and antioxidants, has been proposed as shaping individual fitness. Numerous theories under the field of oxidative stress ecology link sexual selection, reproduction, ageing and survival to disturbances in redox homeostasis. Nevertheless, trustworthy quantification of individual's oxidative status has proven to be more challenging than anticipated. Thus, previous claims about the role of oxidative stress in mediating life history trade-offs have been acquired using non-consistent methods often lacking measurements of either relevant antioxidant or oxidative damage markers.

This thesis aimed to clarify the suitability of some widely used biomarkers of antioxidant protection and oxidative damage for use in studying oxidative stress ecology. In addition, I aimed to highlight the incapability of the current measurement techniques to reliably demonstrate induction of oxidative stress under some ecologically important scenarios.

In order to verify the sensitivity of some popular blood-based antioxidant and oxidative damage markers to oxidative stress, experimental disruption of redox homeostasis was chemically induced in captive greenfinches. From all of the measured biomarkers only erythrocyte glutathione levels and oxidative DNA damage showed sensitivity to manipulation. However, this was evident just among life-threateningly stressed birds, implying either that the used methods, tissue and/or measurement timepoints are unsuitable to detect oxidative stress in a wild passerine, or that outside severe pathophysiologies animals are able to maintain redox homeostasis with intrinsic mechanisms. Unfortunately these intrinsic mechanisms are extremely hard to disentangle using currently available biomarkers of oxidative stress.

Subsequently I aimed to validate a method for quantifying oxidative stress, the d-ROMs test. This method has been both extensively praised and criticised in recent years. I aimed to sort out whether the assay can be modified to selectively determine occurrences of oxidative insult. Unfortunately, it appeared impossible to block the signal from all of the other serum sources that contribute to the d-ROMs test readout without also inhibiting the detection of reactive oxygen metabolites. In addition, it was shown that d-ROMs assay readings from serum samples correlate strongly with serum ferroxidase activity, suggesting that the assay is more suitable for measuring induction of acute phase response than oxidative damage. However, despite this and previous critique the assay is gaining popularity, adding more confusion to studies of oxidative stress ecology.

The costs of mounting an immune response are associated with oxidative stress. I asked whether induction of innate immune response and imposing psy-

chological stress on birds results in disruption of redox homeostasis. Although immune system stimulation imposed energetic costs, only upregulation of antioxidant defences was observed. Such results imply that intrinsic mechanisms may balance increased levels of reactive oxygen species so that oxidative damage to macromolecules cannot be detected. Psychological stress failed to influence any physiological parameter measured. All in all, these results suggest either that oxidative stress has not much to do with these conditions, or that investigating occurrence of oxidative stress under naturally occurring stressors using only some popular blood based biomarkers is misleading.

In order to further elaborate the changes following induction of innate immune response in blood, I subsequently aimed to characterise the full transcriptomic profile of immune challenged greenfinches. As expected, it appeared that the role of most of the up- or downregulated genes could not be established, as they remained unannotated. The available annotation data enabled confirmation that immune stimulation caused cellular rearrangement including upregulation of some avian host defence proteins like avidin, gallinacin and serum amyloid A. Disruption in redox homeostasis was hinted at by induction of some parts of a DNA repair complex. Nevertheless, several oxidative stress related transcripts identified from the dataset showed no change, so that the overall picture was not providing enough support to claim induction of oxidative stress following immune challenge.

Ageing has long been believed to result from oxidative damage to biomolecules. I asked whether age-related trends in blood antioxidant defences or oxidative damage emerge in a long-lived seabird. Although reproductive senescence was evident in the dataset, none of the measured biomarkers exhibited any age-related patterns. Unfortunately no definite conclusions can be reached from these results due to incomplete tissue and biomarker selection. However, such results add further support to the need to revisit the free radical theory of ageing.

In all, this thesis highlights the general insensitivity of some popular blood based biomarkers of oxidative stress to accurately detect disruption in redox homeostasis. In particular the d-ROMs test seems to be unsuitable to measure oxidative status. On the other hand, erythrocyte gluthathione concentration provides some valuable information about the individual's antioxidant defence machinery. However, oxidative stress cannot be measured using only one tissue, timepoint or biomarker. Thus, current measurement techniques are incapable of reliably demonstrating the involvement of oxidative stress in mediating the costs of immune system activation or old age in wild birds.

SUMMARY IN ESTONIAN

Oksüdatiivse stressi mõõtmismeetodite valideerimine ja rakendamine vabalt elavate lindude redoksfüsioloogia kirjeldamisel

Hapnik on mürgine gaas, kuna võib kergesti moodustada erinevaid bioloogilisi struktuure kahjustavaid reaktiivseid radikaale. Seetõttu on organismidel evolutsiooni käigus välja arenenud suur hulk erinevaid antioksüdantseid kaitsemehhanisme, mis võimaldavad kasutada hapnikku energia tootmiseks, vältides seejuures hapniku toksilist mõju. Arvatakse, et oksüdatiivne stress ehk kehas kahjustusi põhjustav tasakaalu puudumine reaktiivsete hapnikuosakeste ja antioksüdantide hulga vahel, põhjustab kohasuse langust. Mitmed teooriad seovad sugulist valikut, sigimist, vananemist ja ellujäämist redoksfüsioloogiaga. Ometi on isendi oksüdatiivse staatuse usaldusväärne mõõtmine osutunud sootuks keerulisemaks kui algselt oodati. Senised tulemused, mis on kinnitanud oksüdatiivse stressi rolli elukäigutunnuste vaheliste lõivsuhete vahendajana, on saadud vastuolulisi meetodeid kasutades. Lisaks pole senistes uuringutes sageli piisavalt hinnatud oluliste antioksüdantide või oksüdatiivsete kahjustuste hulka.

Käesoleva töö eesmärgiks oli analüüsida, kui hästi sobivad mitmed laialt kasutatavad antioksüdantkaitse ja oksüdatiivsete kahjustuste markerid oksüdatiivse stressi mõõtmiseks ökoloogilistes uurimustes. Lisaks kontrollisin oksüdatiivse stressi mõõtmiseks kasutavate meetodite seoseid elukäigu lõivsuhetega erinevates ökoloogilistes kontekstides.

Selgitamaks, kui tundlikud on mitmed laialt kasutatavad antioksüdantkaitse ja oksüdatiivsete kahjustuste biomarkerid oksüdatiivsele stressile, kutsusin rohevintidel (*Carduelis chloris*) keemiliste vahenditega esile oksüdatiivse stressi. Verest mõõdetud biomarkeritest avaldas manipulatsioon mõju vaid punaliblede glutatiooni ning DNA kahjustuste tasemele, ja sedagi vaid eluohtlike oksüdatiivse stressi tasemete juures. Seetõttu võib pidada tõenäoliseks, et kasutatud meetodid, kude ja/või mõõtmisaeg ei sobinud vabalt elavatel värvulistel oksüdatiivse stressi tuvastamiseks. Teisalt võib ka oletada, et kui välja jätta tõsised patoloogiad, suudavad loomad sisemiste mehhanismide abil oma redokshomöostaasi säilitada. Kahjuks on nende sisemiste mehhanismide olekut olemasolevate redoksfüsioloogia mõõtmise meetoditega väga raske täpselt hinnata.

Järgnevalt keskendusin ühe laialt kasutatava meetodi – oksüdatiivseid kahjustusi hindava d-ROMs testi – valideerimisele. Kuigi mitmed tööd on rõhutanud, et d-ROMs test ei pruugi olla sobilik oksüdatiivse stressi marker, on viimastel aastatel seda testi aina enam hakatud just isendi redoksstaatuse määramiseks kasutama. Käesolevas väitekirjas püüdsin välja selgitada, kas ja mil määral saaks d-ROMs testi nii täiendada, et selle testi abil oleks võimalik usaldusväärselt isendi oksüdatiivset seisundit hinnata. Töö tulemusena selgus, et testi tulemusi mõjutavate vereseerumi komponentide inhibeerimine nii, et samaaegselt oleks võimalik täpselt mõõta lipiidsete hüdroperoksiidide kontsentratsiooni, ei ole võimalik. Lisaks selgus, et d-ROMs testi tulemused korreleeruvad

tugevalt vereseerumi võimega rauda oksüdeerida – asjaolu, mis viitab, et test sobib eelkõige põletikuliste protsesside tuvastamiseks, mitte oksüdatiivsete kahjustuste hindamiseks. Seega hägustab d-ROMs testi laialdane kasutamine veelgi võimalust usaldusväärselt hinnata oksüdatiivse stressi rolli isendisiseste lõivsuhete vahendajana.

Oksüdatiivne stress võib kaasneda immuunsüsteemi aktiveerimisega. Seetõttu uurisin, kas kaasasündinud immuunsüsteemi aktiveerimine ja psühholoogiline stress põhjustavad häireid lindude redoksfüsioloogias. Kuigi katseliselt esile kutsutud immuunaktivatsioon oli lindudele energeetiliselt kulukas, kaasnes sellega vaid antioksüdantide kontsentratsiooni tõus. Seega võib järeldada, et organismisisesed regulatsioonimehhanismid võivad tõepoolest olla piisavad, selleks et tasakaalustada suurenenud reaktiivsete osakeste produktsiooni sedavõrd, et biomolekulide kahjustusi pole võimalik tuvastada. Lindudele tekitatud psühholoogiline stress ei olnud piisav, et üleüldse nähtavaid füsioloogilisi muutuseid põhjustada. Nendest tulemustest võib jõuda mitme erineva järelduseni. Esiteks võib olla, et vaadeldud olukordadel ei ole seost oksüdatiivse stressiga. Teiseks on võimalik, et kasutatud oksüdatiivse stressi biomarkerid on ebasobivad looduses esinevate stressoritega kaasneva oksüdatiivse stressi tuvastamiseks.

Täpsustamaks, millised muutused toimuvad lindude veres peale immuunsüsteemi aktiveerimist, võtsin eesmärgiks sekveneerida immuunstimuleeritud rohevintide koguvere transkriptoomi. Suurema osa üles- või allareguleeritud geenide funktsioone ei olnud võimalik tuvastada. Samas võis edukalt annoteeritud transkriptide põhjal järeldada, et immuunaktivatsioon kutsus esile suuremahulise rakusisese ümberkorralduste laine. Muuhulgas suurenes mitmekordselt ka selliste kaitsevalkude nagu avidiin, gallinatsiin ja seerum amüloid A tootmine. Võimalikule oksüdatiivse stressi esinemisele viitas ühe DNA paranduskompleksi mõnede alamosade suurenenud produktsioon. Samas ei viidanud mitmed teised andmestikus esinenud oksüdatiivse stressiga seostatavad transkriptid muutustele redoksfüsioloogias. Seetõttu ei ole võimalik ka vereliblede transkriptoomile tuginedes väita, et immuunaktivatsiooniga kaasneb oksüdatiivne stress.

Oksüdatiivseid koekahjustusi on pikka aega peetud vananemise põhjustajaks. Uurisin pikaealise merelinnu kalakajaka (*Larus canus*) näitel, mil määral korreleeruvad vanusega mõned verest mõõdetavad antioksüdantkaitse ja oksüdatiivsete kahjustuste markerid. Vaatamata sellele, et lindude sigimisnäitajad halvenesid kõrges vanuses, ei korreleerunud ükski mõõdetud oksüdatiivse stressi biomarker lindude vanusega. Nende tulemuste põhjal ei saa siiski teha lõplike järeldusi vananemise ja oksüdatiivse stressi seoste kohta, kuna mõõtsin vaid väikest osa kõikvõimalikest biomarkeritest. Ometi rõhutavad need tulemused veelgi vajadust üle vaadata võimalikud mehhanismid, kuidas vabade radikaalide produktsioon vananemisprotsessiga seotud on.

Kokkuvõtvalt võib öelda, et käesolev töö tõi välja enamike laia kasutust leidvate verest mõõdetavate oksüdatiivse stressi markerite võimetuse usaldusväärselt tuvastada muutusi isendi redokstasakaalus. d-ROMs test tundub olema

eriti sobimatu organismi oksüdatiivse staatuse määramise meetod. Teisalt võib vererakkude glutatiooni kontsentratsiooni mõõtmine anda olulist infot isendi antioksüdantkaitse süsteemi toimimise kohta. Ometi tuleb rõhutada, et oksüdatiivset stressi pole võimalik mõõta, kasutades selleks ainult ühte kude, ajahetke või biomarkerit. Seega ei näita mitmed olemasolevad ökoloogilistes uurimustes kasutust leidvad mõõtmismetoodikad usaldusväärselt, et oksüdatiivne stress vahendab immuunsüsteemi aktiviseerimise või vananemisega seotud kulusid metsikutel lindudel ning uurimistulemustesse, mis on saadud neid meetodeid kasutades, tuleks suhtuda skeptiliselt.

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Conference presentations:

Meitern, R; Sild, E; Sepp, T; Karu, U; Hõrak, P "Effect of chemically induced oxidative stress on measures of oxidative status in greenfinches", 14th International Behavioral Ecology Congress, Lund, Sweden, August 2012, Poster presentation

Meitern, R; Sild, E; Männiste, M; Karu, U; Hõrak, P "Personality relates to measures of oxidative stress in captive greenfinches.", International Ethological Conference for the Study of Animal Behaviour, Newcasltle, Great Britain, August 2013, Poster presentation

Meitern, R; Hõrak, P "Whole blood gene expression profile following an immune challenge in captive greenfinches " Avian Model Systems, Cold Spring Harbour, USA, March 2014, Poster presentation

Meitern, R; Sild, E; Kilk, K; Porosk, R; Hõrak, P "On the Methodological Limitations of Detecting Oxidative Stress: Effects of Paraquat on Measures of Oxidative Status in Greenfinches" 26th International Ornithological Congress Tokyo, Japan, August 2014, Poster presentation

Meitern, R "Monitoring behavior in captivity, computer vision algorithms in use" 34th International Ethological Conference 2015, Cairns, Australia, August 2015, Poster presentation

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Konverentsi ettekanded:

Meitern, R; Sild, E; Sepp, T; Karu, U; Hõrak, P "Effect of chemically induced oxidative stress on measures of oxidative status in greenfinches", 14th International Behavioral Ecology Congress, Lund, Rootsi, august 2012, posterettekanne

Meitern, R; Sild, E; Männiste, M; Karu, U; Hõrak, P "Personality relates to measures of oxidative stress in captive greenfinches.", International Ethological Conference for the Study of Animal Behaviour, Newcasltle, Suurbritannia, august 2013, posterettekanne

Meitern, R; Hõrak, P "Whole blood gene expression profile following an immune challenge in captive greenfinches " Avian Model Systems,Cold Spring Harbour, USA, märts 2014, posterettekanne

Meitern, R; Sild, E; Kilk, K; Porosk, R; Hõrak, P "On the Methodological Limitations of Detecting Oxidative Stress: Effects of Paraquat on Measures of Oxidative Status in Greenfinches" 26th International Ornithological Congress Tokyo, Jaapan august 2014, posterettekanne

Meitern, R "Monitoring behavior in captivity, computer vision algorithms in use" 34th International Ethological Conference 2015, Cairns, Austraalia, august 2015, posterettekanne

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