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PHYSIOLOGICAL CONDITION AND IMMUNE FUNCTION IN GREAT TITS (*PARUS MAJOR* L.): SOURCES OF VARIATION AND TRADE-OFFS IN RELATION TO GROWTH

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers referred in the text by their Roman numerals. Original papers are reproduced with permission from the publishers.

I. Hõrak, P., Jenni-Eiermann, S., Ots, I. & Tegelmann, L. 1998. Health and reproduction: sex-specific clinical profile of Great Tits (*Parus major*) in relation to breeding. *Canadian Journal of Zoology* 76: 2235–2244.

II. Tummeleht, L., Mägi, M., Kilgas, P., Mänd, R. & Hõrak, P. 2006. Antioxidant protection and plasma carotenoids of incubating Great Tits in relation to health state and breeding conditions. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 144: 166–172.

III. Hõrak, P., Ots, I., Tegelmann, L. & Møller, A.P. 2000. Health impact of phytohaemagglutinin-induced immune challenge in Great Tit nestlings. *Canadian Journal of Zoology* 78: 905–910.

IV. Hõrak, P., Tegelmann, L., Ots, I. & Møller, A.P. 1999. Immune function and survival of Great Tit nestlings in relation to growth conditions. *Oecologia* 121: 316–322.

1. INTRODUCTION

1.1. Assessment of individual condition on the basis of physiological health state indices

Individual condition can be defined as the amount of resources available for allocation to fitness-enhancing traits (Lorch *et al.* 2003). Assessment of individual condition is therefore necessary task for field ecologists aiming at understanding why and how individuals differ from each other with respect to their performance. Using of various hemato-serological parameters for diagnostic purposes in poultry and veterinary medicine has a long history (Shapiro & Schechtman 1949; reviewed in Maxwell 1993; Coles 1997). Yet, a systematic interest of avian ecologists for applying those methodologies only emerged in the last decade of the past century (*e.g*. Lochmiller & Dabbert 1993; Gustafsson *et al*. 1994; Jenni-Eiermann & Jenni 1996; Saino *et al*. 1997; Ots *et al*. 1998). These methods, relying on the simple blood sampling techniques, can be used to describe various aspects of individual condition such as immune and nutritional status, stress, antioxidant parameters, or hormone levels. However, understanding the causes of variation of such health state indices is not necessarily simple and straightforward. For instance, the question about how the values of specific condition indices (*e.g*., leukocyte counts) are linked to the state of individual's immune function (*e.g*. Braude *et al*. 1999; Buchanan 2000; Nunn *et al*. 2000; Read & Allen 2000) or how immune function relates to specific parasite resistance (Owens & Wilson 1999; Ryder 2003; Adamo 2004) has raised various controversial issues.

Therefore, to interpret individual condition parameters adequately, more knowledge about the causes and consequences of their natural variation is required. At the time of accomplishing the first papers included in this thesis, some of the above mentioned methodologies were already employed in explaining condition components related to the timing of breeding (*e.g*. Andersson & Gustafsson 1995), physiological changes related to moult and migration (Jenni-Eiermann & Jenni 1994, 1996; Merilä & Svensson 1995), and determination of the physiological costs of reproduction (Gustafsson *et al*. 1994; Hõrak *et al*. 1998). However, the empirical understanding about why individuals differ with respect of the values of particular condition indices and what are the fitness consequences of this variation still remained deficient. Specifically, the questions about which condition parameters are most sensitive to resource limitation and informative in respect of predicting survival were poorly studied.

Based on the background specified above, the first part of this thesis addresses the question about how to assess and interpret variation in individual condition indices during the reproductive event of great tits (Papers I and II). Reproduction is one of most vulnerable stages in birds' life, when the demands of resources are extremely high (*e.g.* Drent & Daan 1980). Also the intensity of parasite infections often increases during the reproductive season of the host as a result of increased parasite transmission and/or increased host susceptibility (Christe *et al.* 2002). General health status during the breeding event should additionally vary with respect to breeding stage, sex (different parental care, physiological contribution), habitat quality and time (diurnal variation). In Papers I and II, health parameters in pre-breeding, incubating and brood rearing great tits were measured and compared. In Paper II, I also tested the applicability of two assays for assessment of the general antioxidant status in wild birds and examined how these parameters are related to the individual levels of carotenoids (as antioxidants of dietary origin) and leukocyte counts (as potential inflammatory markers). Activated immune and detoxification systems can generate excess of reactive oxygen and nitrogen species (ROS/RNS, Halliwell & Gutteridge 1999; von Schantz *et al.* 1999). ROS/RNS are generally unstable and very reactive with a potential to damage nucleic acids, proteins, and lipids if organisms lack sufficient antioxidant protection. Resulting oxidative stress is involved in pathogenesis of possible infectious and inflammatory disorders (*e.g.* Beckman & Ames 1998; Halliwell & Gutteridge 1999). However, the empirical knowledge about the relationships between overall antioxidant protection and other condition indices in wild birds has remained virtually unstudied.

1.2. Immune function and growth: costs, benefits and trade-offs

Immune function, *i.e.*, an ability of an organism to defend itself from internal invaders such as bacteria, viruses or other parasites, is a part of individual's health sate and hence important determinant of its condition. In response to persistent selection pressure by rapidly evolving parasites, the immune system of vertebrates has attained extreme complexity. One common factor that links all classes of immune function is that their operation requires resources that the host could use for some other essential functions such as growth, self-maintenance, reproduction (Sheldon & Verhulst 1996; Norris & Evans 2000; Lochmiller & Deerenberg 2000). Life-history theory assumes that trade-offs are caused by allocation of limited resources to different functions of an organism (Stearns 1992). If immune function is limited by available resources then trade-offs between investments in fitness components and investment in immunity could be important in determining optimal life-history traits. Hence, variability under the amount of resources available for the individual is one factor affecting the occurrence of such allocation trade-offs. Immune-function-based trade-offs should vary also according to the strength of the immune responses themselves. It has been predicted, that (i) strong immune responses will generate trade-offs across a wider range of resource conditions; and (ii) trade-offs will be most easily detected when strong immune responses are expressed under resource-limited conditions (*e.g.* Sandland & Minchella 2003). In favourable environments, hosts can express all life-history parameters, including immune responses, at high levels without observable trade-offs (Reznick *et al.* 2000). Thus, although employment of an immune defence is costly, these costs may often be not visible, because an organism can compensate for the extra demand with an extra intake of resources (Schmid-Hempel 2003).

Recent experiments have shown that activation of immune system may inhibit reproductive success by decreasing the quality of sexually selected traits or decreasing parental care (reviewed in Zuk & Stoehr 2002). Similarly, growth-stress induced immunosuppression has been found among bird nestlings reared in large natural broods (Christe *et al.* 1998) and in experimentally enlarged broods (*e.g.* Saino *et al.* 1997, Nordling 1998; Ilmonen *et al.* 2003; Suorsa *et al.* 2004) with high within-brood competition for resources. However, there is still debate over the occurrence and evolutionary importance of immunological costs in animals (*e.g.* Råberg *et al.* 1998; Schmid-Hempel 2003). Also the "currency" in which the cost of immune response is paid is not clear. It has been argued (*e.g.* Råberg *et al.* 1998; Westneat & Birkhead 1998; Klasing & Leshchinsky 1999; von Schantz *et al.* 1999,) that the costs causing trade-offs between immune function and other demands of organisms are not necessarily paid in terms of energy or macronutrients, but rather as the danger of damaging host tissues due to hyper-activation of the immune system.

Stage of growth can probably be considered as one of the most resource demanding periods in the ontogeny of altricial birds (*e.g.* O'Connor 1977). Thus, the relative cost of immune response during this stage of life may be extremely high (and visible) as it is likely to usurp the resources, which should be used for somatic growth. In the second part of this thesis I address the question about the possible trade-offs between growth and immune function in great tit nestlings (Papers III and IV). Paper III concentrates on testing the costs of induced immune response on growth. In Paper IV the same question was investigated in a complementary way by manipulating the brood size in order to test if immune function is affected by the growth conditions. In order to assess the benefits of elevated immune function, I examined the relationships between immune parameters and survival of nestlings.

2. STUDY SPECIES – GREAT TIT (*PARUS MAJOR* L.)

The great tit is a small (*ca* 19 g), insectivorous short-lived passerine bird that is common throughout the Palearctic region (Gosler 1993). Great tit is facultative double-brooded species that prefers deciduous habitat for breeding (Van Balen 1973; Ulfstrand *et al.* 1981; Lemel 1989; Cramp & Perrins 1993). It is hole-nesting, socially monogamous and sexually dimorphic. In the breeding season, the female lays between 6 and 12 eggs, which are incubated for up to 15 days and the nestlings fledge after a 16–22 days. Both sexes perform the parental care. The yellow ventral plumage coloration of great tits is based on lutein and zeaxanthin (Partali *et al.* 1987; Stradi 1998), two carotenoids that are particularly abundant in lepidopteran larvae (Partali *et al.* 1987) that are the main source of great tits food during the breeding season (Partali *et al.* 1987; Naef-Daenzer *et al.* 2000). This species, like several other small hole-nesting passerines belongs to the favourite research objects of animal ecologists, especially in the context of immunological ecology (*e.g.* Norris & Evans 2000).

3. OVERVIEW OF CONDITION INDICES AND TESTS USED IN THE STUDIES

Leukocytic parameters

Total white blood cell count (WBC) – the number of leukocytes per approximately 10 000 erythrocytes (I, II, III, IV) – Elevated leukocyte number is symptomatic of stress syndrome and inflammatory processes. Usually, leukocytosis is caused by an elevated concentration of heterophils and/or lymphocytes (*e.g.* Dein 1986; Latimer *et al.* 1988; Coles 1997; Ots *et al.* 1998).

Lymphocyte count (I, II, III, IV) – Lymphocytes are immune cells that assist in the recognition and destruction of many types of pathogens. Lymphocyte count serves as indirect measure of cell-mediated immunity. Decreased lymphocyte concentrations may signal stress-induced immunosuppression (reviewed by Hõrak *et al.* 1998), but may also indicate the lack of parasite infections (*e.g.* Ots & Hõrak 1998; Read & Allen 2000). Therefore, interpreting the lymphocyte numbers, knowledge of individuals' background is needed.

Heterophile count (I, II, III, IV) – Heterophils (avian counterparts of mammalian neutrophils) are phagocytosing cells that enter the tissues during the inflammatory response. They are non-specific immune cells, and their lysis during the inflammatory response may be harmful to host tissues (*e.g.* Parslow 1994). Heterophile concentrations increase during inflammatory processes, stress and infections (*e.g.* Jaensh & Clark 2004).

Heterophile lymphocyte (H/L) ratio (I, II, III, IV) – The H/L ratio is widely used stress indicator in birds that has been shown to increase in response to various stressors (*e.g.* Ots & Hõrak 1996; Ilmonen *et al.* 2003). It has been suggested that H/L is more reliable indicator of long-term stress in birds than individual cell numbers, and plasma corticosteroid levels (Gross & Siegel 1983; Maxwell 1993). Since stress is known to suppress immune functions (Sapolsky 1992; Apanius 1998) a high H/L ratio can be interpreted as indicative of immunosuppressive conditions with a concomitant increase in susceptibility to infections (but see Braude *et al.* 1999). Furthermore, the H/L ratio has recently been shown to exhibit high long-term individual consistency (Hõrak *et al.* 2002), indicating its usefulness as a measure of an individual general health status and immunological condition (reviewed by Friedl & Edler 2005).

Hemato-serological indices

Hematocrit (I) – Hematocrit measures the relative amount of red blood cells in total blood volume. It reflects the oxygen carrying capacity of the blood. Low values of hematocrits (anemia) may be caused by bacterial or parasite infections, but may also reflect deficiencies in nutrition, especially the scarcity of some micronutrients such as Fe, Cu, and vitamin B_{12} (Sturkie & Griminger 1986; Jaensh & Clark 2004). On the other hand, hematocrit values can also rise with increased physical exercise (Carpenter 1975; Hõrak *et al.* 1998).

Total plasma protein (I, II) – Consists of albumin and globulins. Decrease in total plasma protein concentration characterises almost all diseases, especially malnutrition. Physical exercise or dehydration results in rise of the level of total plasma protein due to hemoconcentration. Increase in total plasma protein level is associated also with the increase in immunoglobulins (Kawai 1973; Coles 1997; Tatum *et al.* 2000).

Albumin (I, II, III) – Albumin is the largest single fraction in the blood plasma of an healthy individual. Its' main functions are to carry metabolites and other proteins, to act as an amino acid pool in protein synthesis and to serve as an energy resource when glycogen and lipid reserves are depleted. Albumin is essential also for maintaining the osmotic pressure needed for proper distribution of body fluids between intravascular compartments and body tissues. Decrease in albumin concentration in blood plasma accompanies almost any diseases, also malnutrition and blood loss (*e.g.* Kawai 1973; Coles 1997).

γ – globulins (I, III) – This fraction consists mainly of immunoglobulins (Ig) and therefore the concentration of γ – globulins has sometimes been used as an estimate of humoral immune response to different antigens (*e.g.* Kawai 1973; Saino *et al.* 1999). The concentration of γ – globulins increase with both acute and chronic infection (Coles 1997; Tatum *et al.* 2000). Their increase should be compared with any simultaneous lymphocytosis, which may or may not be present (Coles 1997). On the other hand, in case of stress, corticosteroids (stress hormones) can reduce γ – globulin level by suppressing the activity of lymphocytes producing immunoglobulins (e.g. Leonard 1992).

β – globulins (III) – Include carrier proteins, complement, ferritin, C-reactive protein, lipoproteins and fibrinogen, many of which are also acute phase proteins. Increases in β – globulins occur with acute inflammation, inflammatory liver disease, malnutrition, lipemia artefact, systemic mycotic disease and protein losing enteropathies. Decreases occur with hepatic insufficiency and blood loss (*e.g.* Ruff 1982; Grasman *et al.* 2000).

Albumin globulin (Alb/Glob) ratio (I, III) – Plasma albumin level to total globulin level. Lower Alb/Glob ratios occur in acute disease and chronic infectious or inflammatory disease (*e.g.* Kawai 1973, Griminger & Scanes 1986; Coles 1997; Tatum *et al.* 2000).

Triglycerides (I, II) – Triglycerides or fats play an important role in metabolism as energy sources. High levels of plasma triglycerides, besides the superior nutritional status, are associated also with birds gaining mass. Decrease in triglycerides indicates that lipids are less available (Jenni-Eiermann & Jenni 1997). Low triglycerides levels are symptomatic also of a post-resorptive fasting state during which triglycerides from adipose tissues are hydrolysed to free fatty acids and glycerol (Schwilch & Jenni 2001). Hence, triglycerides should be considered together with the other fat metabolites described as next.

Free fatty acids (FFA) (I) **–** Fatty acids can be bound or attached to other molecules, like triglycerides or phospholipids. When they are not attached to other molecules, they are known as “free” fatty acids. Low levels of triglycerides and high levels of free fatty acids and glycerol are expected to characterize fasting and, to some extent starvation (Jenni-Eiermann & Jenni 1998).

Very low density lipoproteins (VLDL) (I) – Lipoprotein subclass. Fatty acids are insoluble in the aqueous medium of the blood and cells and need to be bound to a carrier to be transported. Therefore fatty acids are re-esterified in the liver and transported in the blood as triglycerides packed mainly in VLDL (Jenni-Eiermann & Jenni, 1992). High plasma levels of triglycerides and VLDL are expected to indicate resorption (Jenni-Eiermann & Jenni 1998).

Glycerol (I) – Glycerol is an important component of triglycerides and of phospholipids. When the body uses stored fat as a source of energy, glycerol and fatty acids are released into the bloodstream. Levels of plasma glycerol will decrease in response to lipid metabolism (Jenni-Eiermann & Jenni 1994).

β-hydroxybutyrate (I) – β-hydroxybutyrate is a ketone that is generated from carbohydrates, fatty acids and amino acids. Ketones are elevated in blood after fasting including a night of sleep, in starvation and hypoglycemia. Low β-hydroxybutyrate levels indicate prolonged catabolism of dietary protein, probably linked with a conversion into lipids (Jenni-Eiermann & Jenni 1998; Jenni-Eiermann *et al.* 2002).

Uric acid (I) – Uric acid is the end product of nitrogen metabolism in birds and reptiles. It is considered to have also antioxidant properties (Halliwell & Gutteridge 1999). Levels must be interpreted with caution, because high levels have been linked to a high-protein diet (Hochleithner 1994) and short-term food stress (Jenni-Eiermann & Jenni 1994). Therefore, understanding the meaning of uric acid patterns requires consideration of the other measures of physiological condition.

Plasma carotenoids (II) – Carotenoids are biological pigments acquired from food. Carotenoids are insoluble in water but are fat-soluble. They appear to be extremely important in a variety of physiological processes: free radical absorption, supporting production of the steroid hormones and immunostimulation (Olson & Owens 1998). High levels of plasma carotenoids indicate that the individual is feeding on diet rich of carotenoids.

Measures of total antioxidant capacity (II)

The two methods used for assessment of total antioxidant capacity of plasma – **Total antioxidant status (TAS)** and **Antioxidant potential (AOP) –** are based on the capacity of biological fluids to inhibit redox reaction induced by free radicals. In the presence of antioxidants, the generation of the coloured product is inhibited in proportion to the antioxidants' concentration. Thus, elevated levels of both parameters are indicating the high total anti-oxidant activity of plasma. On the other hand, in some situations increased TAS and AOP levels may reflect compensatory enhancement of antioxidant defences, induced by inflammatory reaction. Such a situation would be analogous to compensatory increase of total antioxidant status subsequent to exercise-induced oxidative stress, as frequently reported in sports medicine (*e.g*. Vider *et al*. 2001; Finaud *et al*. 2006). Both of those assays are widely used in clinical studies (reviewed in Dotan *et al*. 2004).

Phytohaemagglutinin-skin test (III, IV)

The phytohaemagglutinin (PHA) test was originally developed for use in poultry science (Goto *et al*. 1978; Cheng & Lamont 1988). Lectin – phytohaemagglutinin is a compound generated by the red kidney bean (*Phaseolus vulgaris*) where it is believed to serve as a defence against herbivory. PHA is a large molecule (molecular weight 138 000) with a long history of use in immunology, dating back to its original role as an agglutination agent for vertebrate erythrocytes. Like other lectins, PHA is mitogenic to many vertebrate cell types including (but not limited to) T-lymphocytes. PHA skin swelling response can be divided into the two distinct phases. In the first phase of swelling, plasma exudates from surrounding vascular tissue and edema in the injected region. This occurs within 6–12 h after injection and is driven by local innate cell populations (basophils and macrophages) activated by PHAstimulated CD4+ T-cells. The second phase includes an infiltration of additional PHA-sensitive T-lymphocytes, around 24 h post-injection. Activated T-cells secrete some of the cytokines that recruit and activate effectors cells. However, other leukocytes (basophils, heterophils and macrophages) actually effect most of the vasodilation, edema and inflammation of tissue; moreover, these cells secrete additional cytokines, which can promote further infiltration by, or proliferation of, additional leukocytes (reviewed by Martin *et al*. 2006).

Although PHA test only captures part of immune function (Martin *et al*. 2006), it has become popular among field ornithologists because it allows rapid and convenient estimation of in vivo cell-mediated immune response in the wild (Moreno *et al*. 2005). The PHA skin test has been widely used to assess correlation between cell-mediated immunity and body condition (Alonso-Alvarez & Tella 2001; Lifjeld *et al*. 2002), energy expenditure (Martin *et al*. 2003), hormone profiles (Casto *et al*. 2001), brood size (Sorci *et al*. 1997; Saino *et al*. 1997; Moreno *et al*. 1999; Tella *et al*. 2002; Lifjeld *et al*. 2002), feeding rate (Saino *et al*. 1997; Moreno *et al*. 1999), sexual ornaments (Gonzalez *et al*. 1999; Velando *et al*. 2001; Saks *et al*. 2003) and survival (Gonzalez *et al*. 1999; Ardia *et al*. 2003).

4. RESULTS AND DISCUSSION

4.1. Comparison of physiological condition of great tits in pre-breeding and brood rearing periods (I)

To examine how the clinical profile of great tits varies during different stages of the reproductive cycle, I compared condition indices of birds in the pre-breeding period (shortly before the start of egg-laying) and in the middle of nestling period (while feeding 8-days old nestlings). These two periods are likely to differ in respect of both, environmental and physiological stressors imposed to individuals. Brood rearing exerts considerable energetic demands on the parents (*e.g.* Drent & Daan 1980), suggesting a stress accompanying increased work-load to have a negative impact on the individuals' physiological state. On the other hand, nestlings are reared in the period of greatest animal food abundance, which suggests a lack of nutritional constraints. The pre-breeding period, in contrast, should be more limiting nutritionally, because the insect food supplies have not started to develop yet. At the same time, there are no energetic costs related to brood rearing, suggesting minor health impact due to physical burden. Furthermore, there is a reason to expect that sex differences in condition due to differential health impact of sex hormones will emerge especially prominently during the pre-breeding period. Particularly, males in the period of mate and territory acquisition/guarding reveal elevated levels of testosterone and corticosterone (*e.g.* Silverin 1990; Ketterson *et al.* 1991), which due to their immunosuppressive effects, might lead to deterioration of health state (*e.g.* Bohus & Koolhaas 1991; Folstad & Karter 1992). I therefore asked whether the seasonal clinical profiles of males and females differ, predicting that males are in poorer condition than females in the pre-breeding period.

Differences due to breeding stage: Pre-breeding period was not nutritionally more limiting than the nestling period as indicated by the results that in early spring the birds were, on average, 1.5 g heavier and had higher concentrations of total plasma protein, albumin and triglycerides than during the brood-rearing stage (Fig. 2 and 3 in Paper I). The finding that pre-breeding birds had high triglyceride and VLDL levels and low FFA levels (Fig. 3 in Paper I) is surprising because these birds were sampled during the overnight fast, when plasma lipid concentration usually decreases (Jenni-Eiermann & Jenni 1994, 1996). These results indicate that great tits deposit more fat during the pre-breeding stage than during brood rearing, which can be associated with a need to reduce flight costs while provisioning nestlings.

Low concentrations of plasma uric acid and high concentrations of the fat catabolites glycerol and β-hydroxybutyrate (Fig. 3 in Paper I) found in the pre-laying birds are consistent with the pattern typical of overnight-fasted birds. In this time protein is saved and fat reserves catabolised (Jenni-Eiermann & Jenni

1991, 1996). The elevated uric acid concentrations during the nestling period may indicate that the birds are i) either consuming a higher-protein diet or ii) experience food limitation and metabolize endogenous proteins (reviewed in Owen *et al.* 2005). The first explanation seems more likely, given the higher share of caterpillars in the diet during the nestling stage (*e.g.* Perrins 1979).

Brood rearing induced a greater leukocytic stress response than the pre-breeding period, as H/L ratios of both sexes were generally higher during the breeding period (Fig 1, Paper I). H/L ratio is a widely used stress index in poultry, known to increase in response to various stressors, including infectious diseases, starvation, and physiological disturbance (*e.g.* Gross & Siegel 1983; Maxwell 1993). In wild passerines, experimental brood size enlargement has led to increased H/L ratio among both adults and nestlings (*e.g.* Hõrak *et al.* 1998; Ilmonen *et al.* 2003; Suorsa *et al.* 2004). This indicates that H/L ratio reflects stressfulness of the reproductive effort and growth conditions in birds also under natural conditions.

Plasma γ-globulin concentrations were considerably higher during the pre-breeding period (Fig 2, Paper I). Since γ-globulin fraction includes most of the known antibodies involved in immune responses to different diseases (Kawai 1973; Gustafsson *et al.* 1994; Ots & Hõrak 1998; Guemuessoy *et al.* 2004), higher γ -globulins in spring may indicate more severe infections during the pre-breeding period compared to nestlings' period. This explanation would be compatible with the results showing that in captive house sparrows (*Passer domesticus* L.) plasma immunoglobulin concentration was negatively related to survival (Gonzales *et al.* 1999). On the other hand, some researchers have interpreted high plasma antibody levels as indicators of good immune capacity (Saino *et al.* 2001; Christe *et al.* 2001; Morales *et al.* 2004). Hargitai *et al.* (2006) found a positive association between plasma immunoglobulin concentration and body condition of female collared flycatcher (*Ficedula albicollis*). In the study of barn swallows (*Hirundo rustica*) Saino & Møller (1996) showed that males, whose flight cost were increased by experimental tail elongation, failed to increase their γ -globulin level in response to injection with novel antigen, while control birds demonstrated an increase in γ -globulin level. I therefore cannot totally rule out the interpretation that elevated pre-breeding γ – globulin levels in great tits reflect better resource availability for immune defences. If so, this result would point to a possible trade-off between immune response and reproductive effort, so that generally less resources will be allocated into immunoglobulin production during the physically demanding period of brood rearing.

Great tits captured in the pre-laying period had significantly higher hematocrits than the birds captured in nestling period. This could be possibly associated with the lower ambient temperatures in spring, which may increase hematocrits because higher oxygen-carrying capacity is required for thermogenesis (*e.g.* Dawson & Bortolotti 1997).

Sex differences: Most indices of nutritional state did not differ significantly between the sexes, suggesting that there are no consistent differences in nutritional condition between male and female great tits. However, males had remarkably higher β- hydroxybutyrate and FFA levels than females in pre-breeding period (Fig. 3 in Paper I). Elevation of these parameters are characterristic of fat catabolism during fasting (*e.g.* Jenni-Eiermann & Jenni 1994), which suggests that during the overnight fast, males relied more strongly on fat reserves, while females relied more on plasma lipids.

The sexes did not differ systematically considering total plasma protein, albumin, and γ -globulin concentrations (Fig 2, Paper I). However, females had generally lower Alb/Glob ratio compared to the males. Similarly, Ots *et al.* (1998) found sex difference in Alb/Glob ratios of breeding great tits. A low Alb/Glob ratio is a typical symptom of pathological process (*e.g.* Coles 1997; Tatum *et al.* 2000). Kilgas *et al.* (2006a) showed that locally surviving male and female great tits had a higher Alb/Glob ratio during the breeding in the preceding year, compared to non-survivors. Our results may therefore suggest that females had a greater predisposition to infectious diseases than males. Female great tits had more circulating lymphocytes than males in pre-breeding period and more heterophils than males in nestling period. In pre-laying period males had higher H/L ratios than females, while in nestling period the difference was in the opposite direction (Table 1). Seasonal decline in leukocyte numbers, particularly the decline in hererophil number in male great tits might potentially reflect relaxation of environmental stress such as improvement in food availability or increase in ambient temperature, with the progress of the season (Dubiec *et al.* 2005). A sex difference in condition was also showed by Kilgas *et al.* (2006b) in great tits rearing 7 days old nestlings. In their study, females had also lower residual body masses, more heterophils, a higher H/L ratio, higher globulin concentration and lower Alb/Glob ratio than males. Altogether these results indicate that brood-rearing female great tits suffer greater deterioration of health state than males (see also Hõrak *et al.* 1998, 1999). Values of hematocrit did not differ between male and female great tits during the pre-breeding period, while females had remarkably higher hematocrits than males during brood rearing. High hematocrits may indicate elevated oxygen consumption accompanying high workload (Carpenter 1975; Hõrak *et al.* 1998), which again suggest that females work harder than males during brood rearing.

In conclusion, these findings are in accordance with the prediction that the clinical profiles of male and female great tits differ in relation to stage of reproduction due to different commitment of sexes during the reproductive cycle. Brood rearing exerts greater workload and stress to females, and males are more stressed prior to egg-laying.

4.2. Antioxidant protection, carotenoids and indices of general health in incubating females (II)

Oxidative stress – condition that significantly affects general health of an individual – is defined as an unbalance between the production of reactive oxygen and nitrogen species (ROS/RNS), including free radicals, and antioxidant defences (Halliwell & Gutteridge 1999). ROS/RNS are produced by normal metabolism and immune defences, and may cause extensive damage to biomolecules such as nucleic acids, proteins and lipids if the organism lacks sufficient antioxidant capacity. Oxidative stress is also considered as an important source of immunopathology (*e.g*. Beckman & Ames 1998; von Schantz *et al.* 1999; Finkel & Holbrook 2000). To control and neutralise ROS/RNS, organisms have evolved defence systems based on antioxidants, which deactivate reactive species by donating missing electrons. These defences include i) endogenous antioxidants (uric acid, bilirubin, albumin, and enzymes like catalase, superoxide dismutase, glutathione peroxidase) and ii) exogenous antioxidants such as fat-soluble vitamins E and A, and carotenoids (in many passerines also ascorbic acid) that must be obtained from food (Beckman & Ames 1998; Halliwell & Gutteridge 1999). Of all the antioxidants, animal ecologists have paid a disproportionate amount of attention to carotenoids (Lozano 1994; Olson & Owens 1998; von Schantz *et al.* 1999; Møller *et al.* 2000; McGraw 2006). Carotenoid-based visual characters enable individuals to signal their phenotypic and/or genetic quality: if an individual has only a limited amount of carotenoids at its disposal, then it can use them for signals only when it does not need them for maintenance (Lozano 1994). In adult birds, immunostimulatory properties of carotenoids have been repeatedly demonstrated (reviewed in McGraw 2006) while much less is known about the importance of carotenoids as antioxidants.

Here I examined the relationships between plasma carotenoid levels, indices of total antioxidant protection (TAS and AOP), and various hemato-serological health state indices in incubating female great tits in two contrasting (coniferous and deciduous) habitats. To manipulate reproductive effort, four eggs were removed from the half of the clutches during laying to stimulate females to lay additional eggs. Specifically, I addressed the following questions: Under the hypothesis that increased egg-laying effort leads to deterioration of antioxidant protection and general health state (Blount *et al.* 2004; Williams 2005), I predicted that females induced to lay extra eggs reveal lower levels of antioxidant protection, carotenoids, and indices of nutritional state as compared to the un-manipulated females. I also predicted that if dietary carotenoids have any important antioxidant function in incubating birds, then their plasma concentrations should positively correlate with plasma total antioxidant capacity. Additionally, I tested whether plasma carotenoid levels differ between habitat types, expecting higher concentrations in coniferous habitat with presumably higher caterpillar food supply. On the same grounds, I predicted parallel seasonal trends in caterpillar abundance and plasma carotenoid levels. Finally, I asked whether markers of antioxidant protection and plasma carotenoid levels correlate with leukocytic health state indices, predicting negative correlations with markers of inflammation and stress, such as heterophile count and heterophile/lymphocyte (H/L) ratio. I expected negative correlations between markers of antioxidant protection and heterophile hemoconcentration because these inflammatory cells release free radicals, which might deplete the antioxidant capacity (*e.g.* Ames *et al.* 1993; Kogut *et al.* 2002; Costantini & Dell'Omo 2006).

Manipulation of egg-laying effort was unsuccessful since the number of eggs laid by the birds in experimental group did not exceed that of the control group. In accordance with previous studies showing that lepidopteran larvae serve as a main source of dietary carotenoids for breeding great tits (Partali *et al.* 1987; Eeva *et al.* 1998), I found a parallel increases in caterpillar abundance and plasma carotenoid levels during the season (Fig. 1 and 2, Paper II). However, despite the evidently better foraging conditions (Fig. 2, Paper II) and higher body masses in the coniferous habitat, none of the blood parameters differed between habitats (Table 1 in Paper II). Lack of higher carotenoid levels in the coniferous habitat may be explained by the generally lower carotenoid content in the needles of conifers as compared to the leaves of deciduous trees (Czeczuga 1987), so that despite the overall higher food availability in coniferous woods, birds get relatively less carotenoids from the same amount of caterpillars eaten.

Under the hypothesis that carotenoids significantly contribute to antioxidative protection, I predicted that individual plasma carotenoid levels correlate positively with measures of total antioxidativity. Yet no such correlations emerged. In line with our results, serum carotenoid concentration did not correlate with measures of antioxidant protection and serum concentration of reactive oxygen metabolites in a recent study of kestrel (*Falco tinnunculus*) nestlings (Costantini *et al.* 2006). Similarly, lutein supplementation to adult captive zebra finches (*Taeniopygia guttata*) had no direct effect on resistance to of erythrocytes to oxidative lysis (Alonso-Alvarez *et al.* 2004). One possible explanation for these results would be that local actions of carotenoids in specific tissues (*e.g.*, liver) are not reflected in the systemic level, so that plasma total antioxidant capacity is not affected, as shown experimentally in chicken (Woodall *et al.* 1996). Lack of correlation between plasma carotenoids and indices of total antioxidativity can be also reconciled with the results of an extensive meta-analysis of clinical studies of oxidative stress (Dotan *et al.*, 2004), revealing that only under severe pathological conditions all the indices of oxidative stress correlate with each other. However, at present I cannot also totally exclude the alternative explanation, namely that systemic antioxidant properties of carotenoids in birds (except well-established protective effects on embryos and hatchlings – Surai 2002; McGraw *et al.* 2005) may not appear as important as previously thought, at least in situations where general antioxidant balance is not threatened (see also Hartley & Kennedy, 2004).

I predicted that my measures of antioxidant protection correlate negatively with heterophile concentration and H/L ratio. In general, such relationships hold with the exception that at high heterophile concentrations, values of AOP and TAS started to increase again, so that eventually significant U-shaped relationships emerged (Fig. 4, Paper II). Increased amount of circulating heterophils is associated with inflammatory reactions, where they bind and internalize microbes, and subsequently kill them by oxidative burst (Ames *et al.* 1993; Kogut *et al.* 2002). However, the same free radicals released by oxidative bursts may also cause tissue damages if the available antioxidant mechanisms fail to function properly (*e.g.* Terron *et al.* 2003). It is thus possible that the initial decrease in antioxidativity with increasing heterophile hemoconcentration reflects diminishing level of antioxidant protection, induced by inflammatory processes, which are accompanied by increased traffic of heterophils in the blood stream and excess production of ROS/RNS. An increase of plasma antioxidativity at very high heterophile concentrations, in turn, may reflect compensatory enhancement of antioxidant defences, induced by inflammatory reaction. Such a situation would be analogous to compensatory increase of total antioxidant status subsequent to exercise-induced oxidative stress, as frequently reported in sports medicine (*e.g.* Vider *et al.* 2001; Finaud *et al.* 2006). To sum up, a strong association between the measures of antioxidant protection and leukocytic markers of inflammation suggests that measures of antioxidativity deserve attention in ecophysiological studies as potential indicators of immunopathology.

4.3. Does immune challenge affect the growth and health state of nestlings? (III)

Assuming that immune function is resource-limited, it has been expected to compete with other important functions of an organism for the total resource pool, giving rise to trade-offs in resource allocation (reviewed in Sheldon & Verhulst 1996; Zuk & Stoehr 2002; Schmid-Hempel 2003). This study tests whether a trade-off exists between immune defence and growth by examining the physiological impact of an immune challenge in great tit nestlings. I predicted that if such a trade-off exists, nestlings subjected to an immune challenge with a phytohaemagglutinin (PHA, a plant lectin known to induce T-cell mediated inflammatory response) should demonstrate suppressed growth as compared to their untreated siblings if immune response and growth compete for the same resource. In order to better understand the physiological effects of PHA-induced immune challenge, I also examined the effect of PHA injection on the condition indices, such as absolute and differential leukocyte counts and concentrations of plasma proteins. To induce an immune response, half of 8-day-old nestlings in each nest were injected in their wing webs with a PHA. Condition parameters were measured from the blood samples collected immediately before PHA injection and seven days later (*i.e.*, shortly before fledging).

The main findings of this study were that:
1. Immune challenge resulted in increased heterophile hemoconcentration and H/L ratios in the peripheral blood seven days after inoculation.
2. Nestlings treated with PHA did not reveal suppressed growth.
3. The ability of nestlings to produce cutaneous swelling in response to PHA inoculation correlated positively with their subsequent growth during the second half of the nestling period.

The findings of heterophilia and increased H/L ratios in nestlings one week after immune challenge (Fig. 1, Paper III) is of considerable interest because both, the lifespan of heterophils (a few days – *e.g.* Maxwell & Robertson 1998) and the cutaneous hypersensitivity reaction induced by PHA last less than a week (e.g. Stadecker *et al.* 1977; McCorkle *et al.* 1980; Martin *et al.* 2006). Persistent heterophilia in the peripheral blood thus suggests that PHA injection in passerines can induce long-lasting inflammation, such as found for example in chickens injected with turpentine (Latimer *et al.* 1988). To my knowledge, this is the first such a finding in wild birds, which compares interestingly with the recent study of Martin *et al.* (2006). Using biopsies of PHA-injected tissue of house sparrows these authors characterized cellular infiltration into injected wing-web tissue up to 48 h. They found that heterophils appeared at 6 h after the PHA-injection, but then disappeared rapidly, which led the authors to suggest that the wing web swelling was caused by the degranulation of heterophils at the site of injection. The results of the present study suggest that such processes can perhaps induce long-term auto-catalytic systemic inflammatory reactions. Such explanation is also supported by (although marginally) elevated β-globulin concentrations in PHA-injected nestlings (Table 1, Paper III).

Systemic inflammatory reaction is considered as a most expensive aspect of immune responses, both energetically and nutritionally (*e.g.* Klasing *et al.* 1987; Klasing & Leshchinsky 1999). However, despite the lasting effect of the experimental treatment on the profile of immune cells, which suggests the long-lasting inflammation, no effect on nestling growth could be detected (Fig. 1, Paper III). This result differs from those of Klasing *et al.* (1987) and Fair *et al.* (1999), who demonstrated remarkable growth suppression due to immune challenges in domestic chicken and Japanese quail (*Coturnix coturnix*), two species with altricial rapidly growing chicks. In two passerine species, magpies (*Pica pica*, Soler et al. 2003) and blue tits (*Parus caruleus*, Brommer 2004), experimental enhancement of PHA-response by methionin supplementation slowed down the growth rate (though, at least in the magpies, the size and mass at fledging were not affected).

At ultimate level, one of the possible explanations for the lack of growth suppression due to immune challenge might be that nestlings' size is of primary importance for survival and nestlings therefore avoided sacrificing growth for other functions. This explanation complies with the well documented evidence about the positive effect of fledgling mass on their survival (reviewed by Amundsen & Slagsvold 1998; Moreno *et al.* 2005) and also with the results of the Paper IV where fledgling survival was more strongly affected by body mass than lymphocyte count. However, at proximate level such an explanation would be difficult to reconcile with the extended effect of immune challenge upon leukocyte counts, which suggest the lasting inflammatory process. Furthermore, if mounting the immune response against PHA was totally cost-free then it would be difficult to explain why rapidly growing nestlings mounted stronger swelling responses than those who obtained smaller final size at fledging.

Altogether, these findings can be probably best reconciled with the hypotheses proposing that the currency in trade-offs between immune response and other demands of the organism is not necessarily energy or macronutrients (required for growth) but that the trade-offs may be based on immunopathology or oxidative damage (Råberg *et al.* 1998, Westneat & Birkhead 1998, Klasing & Leshchinsky 1999; von Schantz *et al.* 1999 but see Lochmiller & Deerenberg 2000). As argued by Råberg *et al.* (1998), the optimal solution for this state-dependent trade-off is different for animals in good and poor condition, since the former are expected to tolerate higher costs of hyper-responsiveness (that are associated with increased ability to resist pathogens) due to their better ability to repair potential somatic damage. Because both high metabolic rate of the nestlings during growth (*e.g.* Vleck & Bucher 1998) and mounting an immune responses (*e.g.* von Schantz *et al.* 1999) can be associated with increased production of ROS and/or RNS (*e.g.* Ames *et al.* 1993), it might be possible that a positive correlation between the magnitude of the PHA-induced swelling and subsequent growth in great tit nestlings reflects their condition-dependent tactics for avoidance of immunopathology. Such an explanation compares favourably with the recent findings of Costantini & Dell'Omo (2006), showing that Eurasian kestrel (*Falco tinnunculus*) nestlings responded to a PHA challenge with an increase in plasma pro-oxidant concentration and depletion of the total antioxidant defences (although similarly to the present study, growth was not affected). In this context it is also notable that increased heterophile hemoconcentration in incubating female great tits was related to the total plasma antioxidant protection (Fig. 4, Paper II). If the similar relationship holds also for nestlings, one might expect PHA-induced oxidative damages also in the current study.

In conclusion, I found that the response to PHA is resource demanding, but these resources are not reallocated from those used for growth. These results point for the gaps in our understanding of the mechanisms, impacts, and duration, as well as fitness consequences of the inflammatory response induced by PHA injection. Because the measurements of PHA-induced tissue swelling are perhaps the most popular surrogates for immunocompetence in wild birds (Martin *et al.* 2006), I urge the researchers to pay more attention to the long-term physiological impact of PHA-responses.

4.4. Growth conditions, immune function and survival of nestlings (IV)

The aim of this study was to test the complementary prediction of the hypothesis of the trade-off between growth and immune function. In contrast to the Paper III, where immune challenge was induced and its' effect on nestling growth was examined, in this study the growth conditions of nestlings were manipulated and the effects of this manipulation on immune function were examined. On day two after hatching, broods were increased or reduced by two nestlings. To assess the immune function and growth of nestlings, PHA-responses (on day 8) as well as leukocyte counts and body masses (on days 8 and 15) were recorded. To assess the possible fitness benefits of enhanced immune function, the relationships between immune parameters and nestlings' survival were examined both within and between broods under the prediction that non-survivors should reveal symptoms of immunosuppression.

Main findings of this study were that (1) Nestlings from enlarged broods mounted a significantly weaker PHA response and had marginally lower total blood leukocyte and lymphocyte concentrations than nestlings from reduced broods (Table 1, Paper IV). (2) Brood size manipulation affected PHA-response of nestlings most strongly in small clutches (Fig. 1, Paper IV). (3) Intra-brood differences in nestling mortality were unrelated to immune parameters, but (4) nestlings in broods without mortality had a stronger PHA-response, higher concentration of lymphocytes and higher body mass on day 15 than nestlings in broods with mortality.

The simplest explanation to the effect of brood size manipulation on nestling immune function would be that the latter is directly resource limited. An alternative, although not mutually exclusive possibility would be that larger broods may attract more parasites, *e.g.*, because parents are visiting large broods more frequently, increasing the transmission rates, or just because larger broods smell more and parasites can find them easier. In the latter case we would have expected a direct effect of original clutch size on nestling immune parameters. Such an effect was not present for PHA-response, while the lymphocyte count and WBC indeed increased with brood size.

In accordance with the results of this study, the evidence that nestling immune function critically depends on the resource availability is now accumulating. The effect of experimental brood size manipulation on nestling PHA-response has been corroborated in studies of pied flycatchers (Ilmonen *et al.* 2003), barn swallows (Saino *et al.* 1997), Eurasian kestrels (Fargallo *et al.* 2002), zebra finches (Naguib *et al.* 2004), and blue tits (Cichoń & Dubiec 2005; Dubiec *et al.* 2006). On the other hand, no effect of brood size manipulation was found in the study of house sparrows (Bonneaud *et al.* 2003), Eurasian treecreepers (*Certhia familiaris*, Suorsa *et al.* 2004), and tree swallows (*Tachycineta bicolour,* Ardia 2005). The latter results can be probably explained by the efficient parental compensation in response to the increased brood requirements (*e.g.* Ardia 2005). Suppression of the nestling PHA responses due to poor growth conditions was also experimentally demonstrated by male parent removal in great tits by Snoeijs *et al.* (2005). Differently from the present study, Ilmonen *et al.* (2003) and Suorsa *et al.* (2004) found increased H/L ratios among nestlings of increased broods, which indicate that in different species, resource limitation may affect different components of immune function.

The effect of brood size manipulation on PHA-response depended on original clutch size: while the swelling response decreased with clutch size in reduced broods, it increased with clutch size in enlarged broods (Fig. 1, Paper IV). This finding can be interpreted as implying that experimental brood reduction particularly favours pairs with small clutches, while brood enlargement is particularly detrimental to nestlings in small broods. The proximate explanation for this effect is that proportionally greater modification of brood size induced greater differences for nestling immunocompetence. Additionally, this result is consistent with clutch size being condition-dependent: if original clutch size is condition-dependent with large clutches being laid by females in prime condition, then one should expect that experimental reduction of brood size particularly favours pairs in poor condition, while brood size enlargement should have the opposite effect. The mechanism behind this effect could arise from (1) females in better condition lay eggs of better quality, which contain more immunoglobulins, carotenoids or yolk, (2) pairs in better condition provide food of superior quality or quantity for their offspring, and/or (3) such pairs have condition-dependent genetic viability associated with immune responsiveness. Interestingly, in parallel to this study, the positive effect of original clutch size upon the nestlings' PHA-response was also found in a cross-fostering study of American kestrels (*Falco sparverius*, Tella *et al.* 2000).

The prediction that immunocompetence may be crucial for survival found no support by comparison of survived and non-survived siblings. Nestlings that died between day 8 and 15 of the nestling period had significantly lower body mass than their siblings on day 8, but none of the leukocyte parameters were significantly related to intra-brood differences in survival (Table 2, Paper IV). This result differs from those of Christe *et al.* (1998), who showed that non-surviving house martin (*Delichon urbica*) nestlings had lower PHA-responses and higher total leukocyte counts than their surviving siblings. This difference could be probably explained by the fact that the leukocyte counts and PHA response in house martins were recorded when the nestlings were older (day 15 vs. day 8 in the current study), when the immune system of nestlings is more mature and hence more likely to reflect individual differences in fitness.

The relationships between nestling immune function, body mass and survival were also investigated in comparison of broods with and without nestling mortality. This analysis showed that nestlings from broods with no mortality had stronger PHA responses, larger body mass on day 15 and higher lymphocyte count on day 15 than nestlings from broods where at least one young died before fledging (Table 3, Paper IV). Since broods without nestling mortality have superior production of recruits in this particular great tit population (Hõrak 1995), one might conclude that variation in immune function, at least among broods, can contribute to fitness. However, these data do not allow the conclusion that immune parameters are more important than body mass, since the multiple logistic regressions indicated that the effect of body mass on nestling survival cancelled that of lymphocyte count. More clear results in this respect were later found in the study of pied flycatchers (Moreno *et al.* 2005) and blue tits (Cichoń & Dubiec 2005) where nestlings' PHA-response was better predictor of recruitment probability than their body mass. In house martins, fledging survival was more affected by immunoglobulin concentration than by body size, though notably, the PHA response did not predict survival (Christe *et al.* 2001).

More generally, the evidence about the positive relationship between immune parameters and survival in birds seems now to be growing. Recent meta-analysis of survival in relation to some measures of immunocompetence across 12 studies of birds demonstrated relatively high mean effect size, suggesting that immune response generally is a reliable predictor of survival (Møller & Saino, 2004; see also Ardia *et al.* 2003). As regards the indirect leukocytic measures of immune function, nestling survival was negatively correlated with heterophile hemoconcentration in pied flycatchers (Lobato *et al.* 2005) and H/L ratio in great tits (Nadolski *et al.* 2006). These leukocytic parameters were also associated with local survival in adult great tits (Kilgas *et al.* 2006b but see Hõrak & Ots 1998; Dubiec *et al.* 2005), eiders (*Somateria mollissima*; Hanssen 2004), mourning doves (*Zenaida macroura*; Schultz *et al.* 2006) and wood storks (*Mycteria americana*; Hylton *et al.* 2006).

To summarise, in this study I found that immune function of great tit nestlings was affected by their growth conditions and also that differences in nestling survival between broods were related to immune parameters. Although these data did not allow to distinguish between the relative importance of nutritional state and immunocompetence for nestling survival, the strong evidence about benefits of enhanced immune function for survival in wild birds has been accumulated in later studies. Altogether, these findings are consistent with the idea that a trade-off between number and quality of offspring in birds is largely mediated by immunosuppression.

SUMMARY

Evolutionary biologists since Darwin have known that the properties of an individual that are influenced by the external and internal environment, such as health and vigour, affect various components of fitness. Because individuals differ in their ability of acquisition of resources, those in good condition can afford to spend much on several fitness traits while those in poor condition can spend little. However, the empirical understanding about what individual condition is and how it should be measured remained poor until ecologists started to apply clinical health parameters for assessment of health and nutritional state of animals. The main goal of current thesis is to enhance the understanding about the applicability of those simple hemato-serological indices for describing the variation of individual condition of birds in the field.

In some respect, the results were positively surprising, especially as regards the most easily obtainable hematological parameters such as leukocyte counts and differentials. Heterophile hemoconcentration and H/L ratio, which have been previously used as markers of inflammation and stress in poultry, covaried with supposed stressors such as the most demanding stage of breeding (Paper I), immune challenge (Paper III), or reduced antioxidant protection (Paper II) in a predicted manner. Another leukocytic parameter – lymphocyte hemoconcentration was positively associated with nestlings' survival and ability to mount a strong swelling reaction in response to immune challenge with phytohaemagglutinin (PHA) (Paper IV). All these results indicate that the information content of such simple indices is sufficiently high as to be useful for ecological studies. Additionally, this study showed that nestling immune function is sensitive to growth conditions (Paper IV). This is an important assumption of the hypothesis that the trade-off between number and quality of offspring in birds is mediated by immunosuppression, and this finding has been later corroborated in several studies.

On the other hand, this study revealed also some unexpected results, such as long-lasting heterophilia in response to immune challenge with PHA in nestlings (Paper III). If the heterophilia is a hallmark of inflammation and inflammation is energetically most expensive aspect of the immune response, then it remains puzzling why the immune challenge did not suppress growth. This result calls for further attention to the mechanisms and impacts, as well as fitness consequences of the PHA assay, which is currently gaining popularity as a simple proxy for assessment of immunity. Another surprising result of this study was the lack of positive correlation between the plasma carotenoid concentration and total antioxidant capacity, which suggests that at least for the adult birds feeding on naturally carotenoid-rich diet, antioxidant function of carotenoids is not of primary importance (Paper II). This novel result contradicts the popular but largely untested assumption that health benefits of carotenoids at least partially stem from their ability to scavenge free radicals.

To sum up, the results presented in this thesis are encouraging in terms of applicability of easily obtainable hematological parameters for the ecological research. At the same time, this study also pointed to some important gaps in understanding of animal ecologists about the physiological processes leading to the trade-offs involving life-history and signal traits. In any case, application of clinical hemato-serological indices seems to have a great potential for future developments in immuno-ecological research.

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KOKKUVÕTE

Tervisliku seisundi ja immuunparameetrite looduslik varieeruvus ning seosed pesapoegade kasvuga rasvatihastel (*Parus major* L.)

Evolutsioonibioloogia algusaegadest on teada, et isendi tervislik seisund ehk konditsioon mõjutab oluliselt tema kohasuse erinevaid aspekte (nt. viljakust, ellujäämisvõimet, sigimiskordade arvu). Sellest tulenevalt huvitab loomaökolooge, kuidas lihtsate meetoditega välitingimustes kogutud proovide põhjal määrata isendi konditsiooni. Erinevate biokeemiliste ning immunoloogiliste parameetrite määramine vereproovidest on tänaseks kujunemas loomaökoloogiliste väliuurimuste lahutamatuks osaks. Selline lähenemine võimaldab nt. lindude füsioloogilist seisundit iseloomustavaid näitajaid hinnata uurimisobjekte suhteliselt vähe häirides. Kõige lihtsamal viisil perifeersest verest määratavad parameetrid (leukotsüütide kontsentratsioon ning suhtarv ning erinevate plasmavalkude ning metaboliitide kontsentratsioonid) peegeldavad isendi immunoloogilist staatust ja/või üldist tervislikku seisundit. Nimetatud näitajatega on oluliselt seotud ka looma toitumuslik või hormonaalne staatus ning oksüdatiivse stressi tase. Et kõiki neid parameetreid ökoloogilises kontekstis õigesti tõlgendada on vaja rohkem teada tervisenäitajate isenditevahelise varieeruvuse põhjuste ja tagajärgede kohta looduslikes populatsioonides.

Käesoleva dissertatsiooni esimene osa, artiklid I ja II, kirjeldavadki kuidas looduslikus populatsioonis pesitsevate rasvatihaste (kui mudelliigi) konditsiooniparameetrid varieeruvad ajas ja ruumis. Esimeses artiklis võrreldi täiskasvanud rasvatihaste tervisenäitajaid kahes erinevas sigimisfaasis, munemisele eelnenud ajal ja kõige intensiivsemal pesapoegade eest hoolitsemise perioodil. Testiti hüpoteesi, mille kohaselt füsioloogiline stress avaldub erinevates pesitsustsükli faasides emas-ja isaslindudel erineval määral. Selgus, et rasvatihaste tervislik seisund erines oluliselt munemiseelsel ja pesapoegade kasvatamise perioodil. Nende erinevuste põhjuseks võiks olla väliste keskkonnatingimuste ja sigimisfüsioloogiliste protsesside muutumine pesitsusperioodi jooksul. Vastupidiselt ennustatule näitas rasva- ning valgumetaboliitide võrdlus, et tihased ei olnud pesitsuseelsel perioodil (kui loomset toitu napib) halvemas toitumuslikus seisundis kui toidurikkal pesapoegade kasvatamise perioodil. Leukotsüütne stressiindeks (heterofiilide/lümfotsüütide suhe e. H/L) oli pesapoegade kasvatamise ajal üldiselt kõrgem kui munemiseelsel perioodil, mille põhjal võiks oletada, et poegade toitmise etapp on sigimisperioodi kõige kurnamavaks faasiks. Munemiseelsel perioodil oli isaslindude veres lümfotsüütide kontsentatsioon madalam ja H/L suhe kõrgem kui emaslindudel, mis on kooskõlas ennustusega, mille kohaselt isased kogesid pesitsusterritooriumide hõlvamise/kaitsmise ning paariliste pärast konkureerimise faasis emastest tugevamat füsioloogilist stressi. Pesapoegade toitmise faasis olid emastel kõrgemad hematokriti väärtused (mis viitab kõrgele töökoormusele) ja kõrgem

H/L ja kui isastel. Selline oluline sugudevaheline erinevus kliinilistes näitajates viitab sugupoolte vahelisele konfliktile järglaste kasvatamisel.

Artiklis II võrreldi hauduvate emaste rasvatihaste tervisenäitajaid kahes biotoobis, mis erinevad pesitsusaegse toidukülluse poolest. Lisaks varemkasutatud konditsiooni-indeksitele määrati vereplasma karotenoidide sisaldust ning üldist antioksüdantset kaitsevõimet. Karotenoidide funktsioone on loomaökoloogilistes ja ökofüsioloogilistes töödes käsitletud peamiselt sugulise valiku signaaltunnuste kontekstis ja seoses nende immuuno-stimuleerivate omadustega. Samas puudub tänaseni konsensuslik arusaam karotenoidide osast organismi üldise antioksüdantse kaitse moodustamisel ning vereplasma karotenoidide konsentratsiooni ja üldise antioksüdantse kaitse vahelisi seoseid metsikutel lindudel polnud nimetatud artikli kirjutamise ajaks veel kordagi kirjeldatud.

Vastupidiselt hüpoteesile, mille kohaselt vereplasma karotenoidid mõjutavad oluliselt plasma üldist antioksüdantset kaitset, ei leitud hauduvatel rasvatihastel nimetatud kahe parameetri vahel olulist positiivset korrelatsiooni. See tulemus viitab, et karotenoidide antioksüdantne funktsioon looduslikes linnupopulatsioonides võib olla seniarvatust vähem oluline. Vereplasma üldise antioksüdantse kaitse ja leukotsüütsete põletiku indikaatorite (heterofiilide kontsentratsioon ja H/L) vahel ilmnes oluline mittelineaarne seos, mis viitab, et üldine antioksüdantne kaitsevõime võib olla potentsiaalne immuunpatoloogia indikaator.

Dissertatsiooni teine osa (artiklid III ja IV) tegeleb küsimusega, milline on immuunfunktsiooni osa elukäigu lõivsuhetes. Immuunkaitse kasu, hind ja roll mehhanismides, mis seisavad elukäiguteooria lõivsuhete taga on evolutsioonilise loomaökoloogia uurijatele huvi pakkunud juba viimased kümme aastat. Kuna eeldatakse, et immuunsüsteem vajab funktsioneerimiseks ressursse, mida organism saaks samal ajal investeerida ka kasvamisse või sigimisse, siis võib ennustada, et teatud olukordades ohverdatakse immuunfunktsiooni efektiivsus teiste organismi elutähtsate ülesannete nimel ja vastupidi. Nimetatud hüpoteesi testiti rasvatihase pesapoegadel, kelle immuunsüsteemi teatavate komponentide tõhusust hinnati mõlemas töös fütohemaglutiniini (PHA) testi abil. (Taimse lektiini PHA subkutaanse süstimise järel tekkinud paistetusreaktsiooni tugevust usutakse korreleeruvat T-lümfotsüütide proliferatsioonivõimega ning lokaalse põletikulise reaktsiooni tugevusega. PHA test on linnuökoloogide seas järjest populaarsemaks muutuv meetod, kuna seda on välitingimustes lihtne rakendada; samas pole nimetatud protseduuri pikemaajalist füsioloogilist toimet teadaolevalt varem kirjeldatud.)

Artikkel III käsitleb otseselt immuunvastuse hinna teemat. Testiti, kas immuunvastuse esile kutsumine mõjutab rasvatihase pesapoegade kasvu kui üht väga olulist elukäigu parameetrit. Kui immuunvastus konkureerib ressurssidele, mida organism vajab ka kasvamiseks, siis võiks eeldada lõivsuhet ressursi jaotamises immuunfunktsiooni ja kasvamise vahel. Katse tulemustest selgus, et PHA süstimine põhjustas küll muutusi linnupoja leukotsüütsetes vereparameetrites (heterofiilide kontsentratsiooni tõus perifeerses veres), kuid ei mõjutanud pesapoegade kasvu. Sellest hoolimata korreleerus PHA-vastuse tugevus positiivselt linnupoegade järgneva kasvuga.

Seega võib järeldada, et PHA-vastus on küll ressursse nõudev kuid neid ressursse ei võeta kasvamiseks kasutatavate ressursside arvelt. Ootamatuks tulemuseks oli perifeerse heterofiilia esinemine tihasepoegadel veel nädal peale immuunväljakutset, kuna on teada, et nii heterofiilide eluiga kui ka naha hüpersensitiivne reaktsioon PHA-le kestavad vähem kui nädala. Suhteliselt kauakestev heterofiilia viitab, et PHA süstimine võis põhjustada süsteemse pikaajalise põletiku. Sellist pikaajalist vastust PHA süstimisele ei ole seni teadaolevalt lindudel varem kirjeldatud. Antud kontekstis on hämmastav, et PHA süstimine ei põhjustanud kasvu suppressiooni, kuna justnimelt põletikureaktsiooni peetakse immuunvastuse energeetiliselt kõige kulukamaks komponendiks. Need tulemused osutavad, et PHA-vastuse pikaajalised kaasmõjud vajavad immuunökoloogilise uurimistöö kontekstis praegusest rohkemat tähelepanu.

Neljandas artiklis kasutati kasvu ja immuunfunktsiooni vaheliste potentsiaalsete lõivsuhete uurimiseks komplementaarset lähenemisviisi. Manipuleeriti pesakonna suurust (st. pesakonnasisest toidukonkurentsi ja seeläbi kasvutingimusi) ning testiti, kas pesapoegade immuunparameetrid sõltuvad kasvutingimustest. Lisaks testiti, kas immuunfunktsiooni tõhususe indikaatoriteks peetavad tunnused (tugev PHA vastus, kõrge lümfosüütide kontsentratsioon perifeerses veres) ennustavad pesapoegade lennuvõimestumiseelset ellujäämust. Katsest selgus, et kasvutingimused tõepoolest mõjutasid tihasepoegade immuunparameetreid. Tihasepojad, kes olid kasvanud suurendatud pesakondades, tekitasid nõrgemat PHA-vastust, ning võrreldes vähendatud pesakondades kasvanutega oli neil oluliselt madalam üldine leukotsüütide ja lümfotsüütide kontsentratsioon perifeerses veres. Vastupidiselt ennustatule ei erinenud ühe pesakonna ellujäänud ja mitte-ellujäänud pojad üksteisest ühegi immuunparameetri poolest; ootuspäraselt olid ellujäänud mitte-ellujäänutest raskemad. Samas näitas pesakondadevaheline võrdlus, et pesakondades, kus suremust ei esinenud oli tihasepoegade keskmine PHA-vastus tugevam ning lümfotsüütide kontsentratsioon kõrgem, kui pesakondades kus lennuvõimestumiseelset suremust esines. See tulemus on kaudseks tõendiks, et suhteliselt lihtsasti hinnatavad immuunparameetreid nagu leukotsüütide kontsentratsioon ja PHA vastuse tugevus võivad seonduda oluliste kohasuse komponentidega ning seeläbi kätkevad endas ökoloogiliselt väärtuslikku informatsiooni.

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