BLOOD PARAMETERS AS INDICATORS OF PHYSIOLOGICAL CONDITION AND SKELETAL DEVELOPMENT IN GREAT TITS (*PARUS MAJOR*): NATURAL VARIATION AND APPLICATION IN THE REPRODUCTIVE ECOLOGY OF BIRDS

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

I.   Kilgas, P., Tilgar, V. & Mänd, R. 2006. Hematological health state indices predict local survival in a small passerine bird, the great tit (*Parus major*). *Physiological and Biochemical Zoology* 79: 565–572.

II. Kilgas, P., Mänd, R., Mägi, M. & Tilgar, V. 2006. Hematological parameters in brood-rearing great tits in relation to habitat, multiple breeding and sex. *Comparative Biochemistry and Physiology A* 144: 224–231.

III. Kilgas, P., Tilgar, V., Mägi, M. & Mänd, R. Physiological condition of incubating and brood rearing female Great Tits *Parus major* in two contrasting habitats. (Tentatively accepted by *Acta Ornithologica*).

IV. Tummeleht, L., Mägi, M., Kilgas, P., Mänd, R. & Hõrak, P. 2006. Antioxidant protection and plasma carotenoids of incubating great tits (*Parus major* L.) in relation to health state and breeding conditions. *Comparative Biochemistry and Physiology C* 144: 166–172.

V.   Tilgar, V., Kilgas, P., Viitak, A. & Reynolds S. J. 2008. The rate of bone mineralization in birds is directly related to alkaline phosphatase activity. *Physiological and Biochemical Zoology* (in press).

VI. Tilgar, V., Kilgas, P., Mägi, M. & Mänd, R. 2008. Age-related changes in the activity of bone alkaline phosphatase and its application as a marker of pre-fledging maturity of nestlings in wild passerines. *Auk* (accepted).

VII. Tilgar, V., Mänd, R., Ots, I., Mägi, M., Kilgas, P. & Reynolds, S. J. 2004. Calcium availability affects bone growth in nestlings of free-living great tits (*Parus major*), as detected by plasma alkaline phosphatase. *Journal of Zoology* 263: 269–274.

The author's contribution to the papers:

I contributed to planning of the work and data collection in all papers. I performed all data analyses in papers I, II and III and contributed to analyses in V and VI. I was primarily responsible for writing in I, II and III and contributed in IV, V, VI and VII.

1. INTRODUCTION

According to the life-history theory (Stearns 1992), physiological trade-offs arise when animals have limited resources to allocate between competing life-history traits. However, it is a complicated task to determine the appropriate traits that are currently being traded-off or the currency in which the cost is paid (Stearns 1992; Alonso-Alvarez *et al*. 2006). It has been recognized that, to explain life-history variation, we need to understand the physiological mechanisms behind individual and adaptive responses to different environments (reviewed in Ricklefs & Wikelski 2002).

Similarly, in conservation biology, the assessment of individual physiological status in the wild is often the only way to measure the well-being of individuals and populations in a particular environment (reviewed in Wikelski & Cooke 2006; Johnson 2007). For example, due to intensive loss and fragmentation of natural forest habitats, the ecological quality of the remaining plots changes (reviewed in Ewers & Didham 2006). As a result, many bird species have declined in numbers in such human altered habitats (Newton 1998; Debinski & Holt 2000; Groom *et al*. 2006). In the worst case, when changes within a landscape are fast, organisms may not be able to respond fast enough and retain their preference for such habitats (reviewed in Battin 2004; Robertson & Hutto 2006). With such a decrease in the amount of optimal habitat, a larger proportion of individuals are often forced to move to normally avoided or non-preferred habitats (Battin 2004).

Sometimes birds are also attracted to non-preferred habitats by conservationists and researchers who use some wildlife management techniques, *e.g*., providing nest-boxes for secondary cavity-nesting birds (Semel & Sherman 2001; Schlaepfer *et al*. 2002). For example, in our study area, situated in northeastern Europe, it has recently been shown that the occupancy of the nest-boxes by great tits is higher and clutch and egg sizes are larger in fragments of deciduous habitat compared to managed pine forests (Mänd *et al*. 2005). Unexpectedly, the number of offspring and their condition turned out to be higher in coniferous habitat in most years (Mänd *et al*. 2005). However, our knowledge of the individual physiological causes and consequences of habitat choice decisions in human altered mosaics of optimal and sub-optimal habitat is still very limited (*e.g*., Mazerolle & Hobson 2002; Suorsa *et al*. 2003; 2004; Wikelski & Cooke 2006; Johnson 2007).

To describe the environmental effects on different aspects of an individual's physiology, we need to use condition indices that can be easily measured in free-living birds. The use of several blood parameters as indicators of an individual's physiological condition – general health, immune function, stress, nutrition and antioxidant potential has become increasingly popular in avian ecology in the last decade. For example, it has been proposed that reproductive costs may be reflected in an individual's immune system because it interacts

with the general health state of an organism and competes for the resources that can be allocated to reproduction (Gustafsson *et al*.1994; Sheldon & Verhulst 1996; Zuk & Stoehr 2002). Immunological processes interact with an individual's nutritional condition (Lochmiller *et al*. 1993; Saino *et al*. 1997a), reproductive effort (Deerenberg *et al*. 1997; Ilmonen *et al*. 2000) and survival (Hanssen *et al*. 2003; Ardia *et al*. 2003; Hanssen *et al*. 2004; see review in Møller & Saino 2004). Also the antioxidant defense system has been proposed to be one mechanism connecting life-history traits by mediating trade-offs between them (von Schantz *et al*. 1999; Wiersma *et al*. 2004). Increased metabolic investment associated with reproduction, as well as an activated immune system can generate an excess of reactive oxygen and nitrogen species – ROS/ RNS (Alonso-Alvarez *et al*. 2004a; Wiersma *et al*. 2004; Bertrand *et al*. 2006). ROS/RNS are generally unstable and very reactive with a potential to damage nucleic acids, proteins and lipids (Halliwell & Gutteridge 1999). To control and neutralize ROS/ RNS, organisms evolved defense systems based on antioxidants. In mammals it is well known that oxidative stress can be associated with different health problems (Halliwell & Gutteridge 1999). However, the connections between antioxidant protection and health indices have remained virtually unstudied in free-living birds.

Sometimes the variation in the values of condition and health indices in wild animals is difficult to interpret (Read & Allen 2000; Hõrak *et al*. 2002; Adamo 2004), because the meaningfulness of the changes in particular indices is largely unknown. It is often necessary to use several condition indices in parallel in order to understand complex changes in an individual's physiology (Matson *et al*. 2006). Therefore it is also important to know how different condition indices are related to fitness components such as survival and reproduction. Still, so far only very few studies have examined connections between widely used hematological condition indices and survival in adult birds (Hõrak & Ots 1998; Gonzalez *et al*. 1999; Hanssen *et al*. 2003; Verhulst *et al*. 2004). Similarly, until quite recently most avian studies have assumed that reproductive costs consist mainly of the costs of chick rearing (reviewed in Monaghan & Nager 1997; Williams 2005). However, recent investigation has revealed that egg production is also relatively costly and can lead to negative changes in female condition (Oppliger *et al*. 1996; Kalmbach *et al*. 2004; reviewed in Williams 2005) and survival (Visser & Lessels 2001). We still know very little about the physiological consequences of egg laying effort in free-living birds.

To use condition indices effectively on free-living birds we also need to know the natural causes of variation of those indices (Fair *et al*. 2007). Previously it was mentioned that individuals in different populations could differ in regard to their health and condition. Different hematological variables of birds can vary between habitats, especially in relation to habitat structure and trophic conditions (Bańbura *et al*. 2007 and references therein). The spatial variation of individual condition may increase if habitat quality changes over

time (Block & Brennan 1993). Therefore it is important for researchers to know how different condition indices change in time in order to use appropriate measures to describe different temporal changes in habitats (Johnson 2007). This can be a problem in the case of multiple brooded species. For example, it has been shown that changes in the caterpillar food abundance during the breeding season are different in coniferous and deciduous woods (van Balen 1973; Eeva *et al*. 2000; Rytkönen & Orell 2001). Also the relative reproductive investment of great tits into the first and the second breeding attempts can differ between habitats (Mägi & Mänd 2004; Pimentel & Nilsson 2007). However, very few studies in birds have considered changes in different aspects of adult body condition between first and second broods (*e.g*., Christe *et al*. 2002). In addition, the condition indices should also vary with respect to stage within one breeding event, because of the seasonal changes in energetic requirements, hormones and climatic conditions. For example, the decrease of body mass during breeding is a commonly observed phenomenon in female birds (*e.g*., Freed 1981; Merilä & Wiggins 1997; Cichoń 2001). Much less is known about changes in physiological parameters throughout the different reproductive phases in free-living birds (*e.g*., Hõrak *et al*. 1998a; Vanderkist *et al*. 2000; Kern *et al*. 2005; Gayathry & Hedge 2006; Fair *et al*. 2007). Moreover, there are almost no studies examining the temporal changes in physiological condition indices of breeding birds simultaneously in different quality habitats.

The condition and health state of individuals during reproduction have also been shown to differ between males and females. Generally males tend to suffer more from parasitic infections and have reduced immune responses compared to females (Poulin 1996; Klein 2000; Zuk & Stoehr 2002; but see McCurdy *et al*. 1998). These sex differences can arise from the different life histories of sexes or from the immunomodulating effects of sex hormones (Zuk & Stoehr 2002). Also spatial and temporal variation in body condition can be sex-specific (Block & Brennan 1993; Hõrak *et al*. 1998a). However, there is still only a limited amount of data available from natural populations.

It is known that many physiological parameters in nestlings change with age. That gives us the possibility to use physiological parameters to describe different aspects of nestlings' growth. For example, the traditional way to describe nestlings' maturity has been to measure the final body size or body mass of fledglings. However, it is known that chicks that experienced unfavorable conditions early in life can often compensate their retarded growth by increasing their growth rate when conditions improve (Metcalfe & Monaghan 2001). They can also delay their fledging time to catch up with their nest mates in size. Minerals are deposited in the bones more slowly than the bones increase in length, and the calcium content of the body increases until several weeks after fledging (Ricklefs 1975; Austin & Ricklefs 1977; Dutta *et al*. 1998). Since the morphological parameters of the young are usually measured just before fledging, when chicks have achieved full structural size, it is possible that

fledglings of similar body size are actually at different stages of skeletal development. Revealing such subtle differences in skeletal ossification would provide valuable information. For example, the length of the nestling period of passerines can vary widely within species and is greatly affected by local environmental factors (*e.g.*, Keller & van Noordwijk 1994). Thus, depressed growth rate in particular broods may result in a prolonged nestling period, potentially reducing the survival of chicks before fledging (Sullivan 1989; Keller & van Noordwijk 1994; Gebhardt-Heinrich & Richner 1998). Alternatively, under circumstances of premature forced-fledging, chicks from broods with slow average growth rate may suffer costs (*e.g.*, fledglings with shorter wings are weaker flyers; McCarty 2001). Hence, survival of young with delayed development of bones may be reduced. As it is impossible to discriminate between nestlings at different developmental stages solely from gross skeletal traits such as bone lengths, using other developmental measures is necessary. Alkaline phosphatase (ALP), namely its bone isoform (bone-ALP) is a candidate for examining the skeletal development of nestlings (see Material and Methods). Still, only a few studies have measured bone-ALP enzyme in birds (*e.g.*, Tilgar *et al*. 2004; Smits *et al*. 2007).

The general aim of this thesis was to describe the natural variation of several blood parameters as indicators of condition and development and to use them in the study of reproductive ecology in great tits. Throughout the thesis blood parameters are used in parallel with traditional morphological indices such as body mass and tarsus length. The first part of the thesis examines the condition indices in breeding adult great tits. The following questions are asked: Are the condition indices measured during breeding related to survival (**I**) and reproductive effort (**II**, **III**, **IV**)? Is there variation in adult condition indices in relation to sex (**II**), habitat type (**II**, **III**, **IV**) or stage of reproduction (**II**, **III**, **IV**)? How is the general antioxidant potential related to other condition indices (**IV**)?

The second part of the thesis examines the variation in the level of alkaline phosphatase in the plasma of great tit nestlings. The following questions are asked: How do alkaline phosphatase levels relate to skeletal mineralization (**V**) and age of nestlings (**V**, **VI**)? Do alkaline phosphatase levels reflect growth conditions (**VII**) and chick maturity (**VI**, **VII**)?

2. MATERIAL AND METHODS

2.1. Study system and study species

The study was conducted in the surroundings of Kilingi-Nõmme (58° 7' N; 25° 5′E) in SW Estonia in 2000–2005. Various forest habitats in the 50-km^2 study area can be divided into two contrasting types – deciduous and coniferous woods (see Mänd *et al*. 2005 for detailed scheme). Deciduous forest fragments grow on fertile soils that have a rich deciduous understorey. The most common tree species are grey alder (*Alnus incana*) and silver birch (*Betula pendula*). The coniferous habitats are typical continuous managed pine forests on nutrient-poor sandy or peat (in the lower parts of the terrain) soil. The dominant tree species is Scots pine (*Pinus sylvestris*), which sometimes occurs in mixed stands of Norway spruce (*Picea abies*) on sandy soils or downy birch (*Betula pubescens*) on peat soils.

Previous studies in the same study area have found that during the nestling period of the first breeding attempt, the numbers of larvae and adults of lepidoptera were higher in the deciduous than in the coniferous habitat (Mänd *et al*. 2005). Also the density as well as the mean number of species of snails was significantly higher in the deciduous than in the coniferous habitat (Tilgar *et al*. 1999).

The great tit (*Parus major*) is a small (*ca*. 19 g) insectivorous short-lived passerine bird that is common throughout the palearctic region (Perrins 1979; Gosler 1993). The great tit inhabits various woodlands, yet prefers deciduous habitat for breeding (van Balen 1973; Mänd *et al*. 2005). It is a facultative double-brooded species, and in our study area 40–70% of females lay a second clutch during the same breeding season (Mägi & Mänd 2004).

2.2. General field methods

Great tits bred in wooden nest-boxes with cavity measurements of 11×11×30 cm and an entrance diameter of 3.5–4.0 cm. Nest-boxes were mounted on tree trunks at a height of 1.5–2.0 m. The nest-boxes were arranged in lines, and each line consisted of some tens of nest-boxes. Distance between neighboring nest-boxes was 50–60 m. Old nest material was removed every year prior to the start of the breeding season.

Nest-boxes were checked regularly to record basic breeding parameters – laying date for the first egg, clutch size, hatching date, brood size, the number of fledglings and occurrence of second broods.

Nestlings were weighed and their tarsus length was measured on the 8th (**VI**) and 15th (**I**, **II**, **III**, **VI**, **VII**) day post-hatch. In addition, blood sample for

plasma alkaline phosphatase activity measurement was taken on the 8th (**VI**) and 15th (**VI**, **VII**) day post-hatch. For paper **V** the body measurements were obtained and blood samples were taken on days 5, 7, 9, 13, 15 and 17 post-hatch. In addition, nestlings were killed by terminal anesthesia, dissected, cleaned with the help of larder beetles (*Dermestres lardarius*) and the calcium content of bones was analyzed by atomic absorption spectrophotometry (**V**). In paper **VI**, the occupied nest-boxes were visited every day after day 15 post-hatch, to determine the actual day of fledging.

Male (**I**, **II**) and female (**I**, **II**, **III**) adults were captured on their nests when nestlings were 8–15 d old. In papers **III** and **IV**, females were captured on the fifth day of incubation. All captured adults were banded with individual rings, their weight and tarsus length was measured and a blood sample for health parameter analyses was taken. In paper **I**, recapture rate was used as an estimate of local survival (Tinbergen & Verhulst 2000; Stjernman *et al*. 2004). See original papers for more details.

Caterpillar abundance (**IV**) in both habitats was monitored by frass-fall method (*e.g*., Seki & Takano 1998).

2.3. Experimental manipulations

To manipulate egg-laying effort (**IV**), four eggs were removed from half of the clutches during laying to stimulate females to lay additional eggs (*e.g*., Visser & Lessels 2001). To standardize the incubation effort (Visser & Lessels 2001; Hanssen *et al*. 2005), the size of each experimental clutch was equalized to its control pair member after clutch completion.

To manipulate calcium availability during nestling growth (**VII**), experimental nests were supplemented with calcium-rich material (chicken eggshell fragments) during the nestling period (*e.g*., Graveland *et al*. 1994; Tilgar *et al*. 1999). Control nests were left unsupplemented. See original papers for more details.

2.4. Blood parameters used

Lymphocyte concentration (**I**, **II**, **IV**) − Lymphocytes are immune cells that assist in the recognition and destruction of many types of pathogens. The lymphocyte concentration in peripheral blood can be used as an indirect measure of cell-mediated immunity. However, sometimes its values are difficult to interpret (*e.g*., Read & Allen 2000). Decreased lymphocyte concentrations may signal stress-induced immunosupression (Hõrak *et al*. 1998b), but may also indicate a lack of parasite infections (Ots & Hõrak 1998).

Heterophile concentration (**I**, **II**, **IV**) – Heterophils are non-specific phago-cytosing cells that enter the tissues during inflammatory processes. The production of reacive oxidants by heterophiles during inflammation can be harmful to host tissues (Surai 2002). Heterophile concentrations increase during inflammatory processes, stress and infection (Coles 1997).

Heterophile lymphocyte ratio (H/L ratio) (**I**, **II**, **IV**) – The H/L ratio is a widely used indicator of stress and it increases in response to various stressors like infectious diseases, starvation and reproductive effort (Gross & Siegel 1983; Ots & Hõrak 1996; Moreno *et al*. 2002).

Hematocrit (**I**, **II**, **III**) – Hematocrit measures the relative amount of red blood cells in total blood volume. It reflects the oxygen carrying capacity of the blood. Its low values (anemia) are indicative of bacterial or parasite infections, but also reflect deficiencies in nutrition, especially the scarcity of some micronutrients such as Fe, Cu and vitamin B_{12} (Svensson & Merilä 1996; Coles 1997). Hematocrit value can also rise with hard physical exercise (Saino *et al*. 1997b) and decrease in egg laying birds (reviewed in Williams *et al*. 2004).

Albumin (**I**, **II**, **III**, **IV**) – Albumin is a plasma protein synthesized by the liver using dietary proteins. It acts as a carrier of metabolites, an amino acid pool in protein synthesis, and also as an energy source when glycogen and lipid reserves are depleted. A decrease in albumin concentration in the blood plasma accompanies almost all diseases and is also a strong indicator of malnutrition (*e.g*., Kawai 1973; Hõrak *et al*. 2004).

Globulins (**I**, **II**, **III**) – The gamma globulin fraction of the serum includes most of the known antibodies involved in the immune response to protozoan, bacterial and viral infections. The concentration of gamma globulins increases with both acute and chronic infections. The beta globulins include a variety of proteins and also contain several acute-phase proteins. An increase in beta globulin concentration in blood plasma is expected with acute inflammation (Coles 1997).

Albumin/globulin ratio (Alb/Glob ratio) (**I**, **II**, **III**) – Healthier individuals in general have higher Alb/Glob ratios (Kawai 1973; Coles 1997; Hõrak *et al*. 2004).

Triglycerides (**I**, **II**, **III**, **IV**) – Triglyceride concentration in blood plasma reflects the rate of lipid transport into adipose tissues and can be considered as an estimate of the amount of lipids absorbed in the few hours before blood sampling (*e.g*., Jenni-Eiermann & Jenni 1997). High triglyceride levels are also associated with mass gain (*e.g*., Cerasale & Guglielmo 2006).

Plasma carotenoids (**IV**) – Carotenoids are lipid-soluble hydrocarbons that generate a range of red, orange, and yellow hues in avian feathers and bare parts. They are acquired from food. In addition to colouration they are used as antioxidants and immunomodulators (reviewed in McGraw 2006).

Total antioxidant status (TAS) and **antioxidant potential (AOP)** (**IV**) – Both methods were used to assess the total antioxidant capacity of plasma. They are based on the capacity of biological fluids to inhibit redox reaction induced by free radicals. Elevated levels of both parameters indicate the high antioxidant activity of the plasma (reviewed in Surai 2002).

Alkaline phosphatase (total-ALP) (**V**, **VI**, **VII**) – Alkaline phosphatases are a family of glycoproteins that are present in blood plasma in several isoforms. The two major and diagnostically most relevant isoforms of this enzyme are liver-ALP and bone-ALP (*e.g.*, Genge *et al*. 1988; Romagnoli *et al*. 1998). **Bone-ALP** is synthesised by osteoblasts in the bone tissue and it has been suggested to be a highly specific marker for skeletal calcification and growth in both mammals and birds (Kubota *et al*. 1981; Price 1993; Tilgar *et al*. 2004). Active bone growth is accompanied by increased activity of bone-ALP in the plasma, and after completion of skeletal development, the level of this enzyme decreases quickly (Price 1993). **Liver-ALP** activity is not directly related to skeletal development, but reflects other aspects of metabolism (Alvaro *et al*. 2000; Watkins *et al*. 2000).

The laboratory procedures used to assess blood parameters are described in original papers. In addition, **body mass** was used as an indicator of overall condition. In papers **I**, **II**, **III** and **IV** body mass of adults was corrected for structural size in the models. In the thesis, such body mass is called size-corrected body mass. In papers **V**, **VI** and **VII** the uncorrected body mass of nestlings was used.

3. RESULTS AND DISCUSSION

3.1. Blood parameters as measures of physiological condition in adult great tits

3.1.1. Connections with survival

Although it has been shown that there exists a link between immune function and survival in birds (reviewed in Møller & Saino 2004), much less is known about whether widely used blood parameters can be used to detect such a link in free-living birds. To study the relationship of different condition indices with survival, adult great tits were sampled during brood rearing and their survival to next year was monitored. It was found that the local survival of adult great tits was related positively to their plasma Alb/Glob ratio and negatively to their globulin concentration and H/L ratio (**I**). Low values of Alb/Glob ratio are known to reflect various kinds of infections and/or symptoms of malnutrition (Kawai 1973; Coles 1997). An increase in plasma globulin concentration is expected after injury or pathogenic challenge (Kawai 1973; Ots & Hõrak 1998; Gümüşsoy *et al*. 2004). H/L ratio is a stress indicator, which is known to inc- rease in the presence of various stressors like infectious diseases or starvation (Gross & Siegel 1983). In great tits, it has also been shown that parents who make intense reproductive effort have high H/L ratios (Ots & Hõrak 1996; Hõrak *et al*. 1998b). Thus, my results indicate that non-surviving individuals may experience higher pathogenic challenge and are more stressed at the end of the breeding session compared to surviving birds.

Local survival of male great tits was also positively related to lymphocyte concentration, while the value of this trait did not contribute significantly to survival of females (**I**). Lymphocyte numbers in the peripheral blood of birds are known to decrease in response to different stressors like food scarcity (*e.g*., Totzke *et al*. 1999) and after intense physical activity (Hõrak *et al*. 1998b). Thus, it is possible that males with higher lymphocyte concentrations and greater survival probabilities had more resources to allocate into immunity. Although I do not know exactly what mechanisms are behind the observed sex difference in the relationship between lymphocyte concentration and survival, the answer probably lays in different endocrine and immune system interactions in males and females (Folstad & Karter 1992; Klein 2000; Barbosa & Moreno 2002). Male birds generally have higher concentrations of testosterone during breeding, compared to females and high concentrations of testosterone can sometimes suppress immunity (Verhulst *et al*. 1999; Peters 2000).

The effect of health on survival seemed to be unrelated to reproduction, since reproductive parameters (number of fledgings, fledging success, nestling

body mass and tarsus length, occurrence of second broods) had no effect on adult survival (**I**).

A positive association between aspects of immune function and survival need not imply that immunological state *per se* affects survival, because it is possible that both traits are condition dependent and are affected by condition simultaneously (Sheldon & Verhulst 1996). However, the local survival of adults was not influenced by size-corrected body mass or triglyceride and albumin concentrations (**I**) – the traits reflecting mainly the nutritional status of an individual (Coles 1997; Jenni-Eiermann & Jenni 1997). Also hematocrit was unrelated to survival (**I**). Moreover, inclusion of body mass in models simultaneously with blood parameters did not alter the results (**I**). Therefore, I suggest that the observed relationships indicate a causal link between some aspects of immune condition during breeding, and future survival. These results also imply that both, leukocytic parameters and plasma protein profiles can be used in future studies to detect health problems in free-living birds.

3.1.2. Connections with reproductive effort

If individuals pay the cost of reproduction in terms of deleterious effects on their physiological condition, then intense reproductive effort should result in poor condition. Unfortunately, the attempt to manipulate egg-laying effort during second broods was unsuccessful, as the number of eggs laid by the experimental group did not exceed that of the control birds. It was therefore anticipated that physiological variables recorded during the fifth day of incubation did not differ between manipulated and control birds (**IV**). Another study in the same population using the same manipulation technique conducted during the first breeding attempt yielded an increase in clutch size by one egg (Mänd *et al*. 2007). It is possible that during the second breeding attempt the birds refrained from increasing their reproductive effort due to time constraints (Verboven & Verhulst 1996; Visser *et al*. 2003).

I also found no correlations between physiological indices and clutch size (**III**) or number of fledglings (**II**). Also the parents' decision to start a second clutch did not depend on any condition indices measured at the end of the first breeding attempt (**II**). This result coincides with the results reported for great tits by other authors who have used body mass as a condition indicator (Tinbergen 1987; Smith *et al*. 1987; Lindén 1988; Mägi & Mänd 2004; but see de Laet & Dhondt 1989). However, it has been found in barn swallows (*Hirundo rustica*) that the frequency of second clutches was higher in ecto-parasite free nests (Møller 1993) and in adults with higher natural antibody level (Møller & Haussy 2007). Based on my results, it seems unlikely that the probability of double breeding in great tits is a function of their condition or immunological state.

3.1.3. Sex differences

It was found that during chick rearing period, female great tits had lower size-corrected body masses, more heterophiles, a higher H/L ratio, higher globulin concentration and lower Alb/Glob ratio, and during the first breeding attempt also higher hematocrit than males (**II**). Thus, females in my study system seem to be in a poorer body condition, more stressed and probably also immuno-logically more challenged than males during brood rearing. At the same time, no sex differences were found in lymphocyte, albumin and triglyceride concent-rations (**II**).

It has generally been shown that male birds tend to suffer more from parasitism and have reduced immune responses compared to females (Poulin 1996; Klein 2000; Moreno *et al*. 2001; Zuk & Stoehr 2002; but see McCurdy *et al*. 1998). However, as shown in several bird species, very often there are no sex differences in various hematological health state indices (Aquarone *et al*. 2002; Hauptmanová *et al*. 2002; Masello & Quillfeldt 2004; Sánchez-Guzmán *et al*. 2004; Fair *et al*. 2007). It seems thus that sex differences in the physio-logical parameters can differ considerably between species. However, Hõrak, Ots and others, who worked with a great tit population relatively close to my study area, received very similar results – the females caring for first broods had higher hematocrits, higher heterophile concentration and H/L ratios and lower Alb/Glob ratios than males (Hõrak *et al*.1998a; Ots *et al*. 1998).

According to my results, most of the observed sex differences, except for the hematocrit, seem to persist also during the second breeding attempt. Thus, the reasons causing these sex differences are probably the same in both breeding attempts. It is possible that either different endocrine profiles between sexes (Klein 2000) or the greater contribution of females to offspring rearing (Sanz *et al*. 2000; but see Lessels *et al*. 1998) are behind the observed sex differences in condition indices. One can also hypothesize that sex differences in condition indices are partly caused by egg laying and incubation costs in females (Visser & Lessels 2001; Kalmbach *et al*. 2004; Williams 2005). Also brood size, being larger in first broods compared with second broods (Mägi & Mänd 2004), could explain why a significant sex difference in hematocrit was found only during first broods (**II**). This sex difference may be an indication of females' greater oxygen consumption resulting from more intense parental care compared to males.

3.1.4. Habitat-related variation

In my study area, it has recently been shown that during first broods the occu-pancy of the nest-boxes by great tits is higher and clutch and egg sizes are larger in the fragments of deciduous habitat compared to managed pine forests (Mänd

et al. 2005). Unexpectedly, the number of offspring and their condition turned out to be higher in coniferous habitat in most years (Mänd *et al.* 2005). Knowing the physiological condition of parents in both habitats during different reproductive phases can potentially shed light on the possible causes of such a paradox.

Incubation period: When incubating first clutches, great tit females in coniferous habitat had higher size-corrected body mass, hematocrit, plasma Alb/Glob ratio and albumin concentration, compared to those breeding in deciduous habitat (**III**). At the same time, no habitat-associated differences were found in tarsus length, beta and gamma globulin concentrations and triglyceride concentrations (**III**). My results indicate that females were in a better nutritional condition (see Material and Methods) during incubation in coniferous than in deciduous habitat. This finding is of particular interest in the light of numerous studies that clearly show that the nest-box occupation rate by great tits is higher in deciduous habitat than in coniferous habitat (*e.g.*, van Balen 1973; Klomp 1980; Cramp & Perrins 1993; Mänd *et al.* 2005; **III**). The fact that females also had smaller clutches in coniferous than in deciduous habitat (**III**) may lead to the assumption that the observed habitat-specific variation in female condition is just the result of a lower investment in egg production (Williams 2005) or of lower incubation costs (Hanssen *et al.* 2005) in coniferous habitat. However, since the habitat differences in condition indices persisted even when clutch size was included in the model (**III**), I have reason to believe that the effect of habitat on female condition is independent of the egg-laying and incubation costs.

I cannot exclude the possibility that the habitat-related differences in female condition already existed before egg laying. This could be possible if only females in superior condition usually settle into non-preferred coniferous habitat. However, since individuals in prime condition are generally expected to settle into preferred habitats (Newton 1998; Stamps 2006), more research is required to clarify this question.

It is also possible that foraging conditions early in the season are better in coniferous habitat. Similarly to Rytkönen & Orell (2001) in Finland, Mägi (2007) has found no differences between deciduous and coniferous habitat in caterpillar biomass during the incubation period of the first clutches for our study area. However, as the breeding density of great tits in our study area is much higher in young deciduous habitat fragments compared to coniferous habitat (Mänd *et al.* 2005; **III**) it is possible that the *per capita* amount of food is actually higher in coniferous habitat.

In another year the condition of incubating females during second clutches was measured. However, there were no differences in physiological parameters between habitats, although females weighed more in coniferous habitat (**IV**). The caterpillar abundance also tended to be higher in coniferous habitat (**IV**).

Thus feeding conditions appeared to be slightly better in coniferous habitat during the incubation of second clutches.

Brood rearing period: It was found that great tits breeding in coniferous habitat had higher hematocrit, higher Alb/Glob ratio and lower total globulin concentration and during the first breeding attempt, also lower lymphocyte concentration than those breeding in deciduous habitat at that time (**II**). Also heterophile concentration was lower in coniferous habitat compared to deciduous habitat, although there were also yearly differences in the effect size (**II**). At the same time, no habitat differences were found in size-corrected body mass, triglyceride and albumin concentrations (**II**). Based on these results, I suggest that great tits breeding in coniferous habitat are in a superior health state, compared with those breeding in deciduous habitat. It is possible that higher breeding density in deciduous habitat fragments compared to sparsely occupied coniferous habitat (Mänd *et al*. 2005; **III**) has caused an increased intraspecific interference (reviewed in Newton 1998). It is unlikely however that the observed habitat differences in parental condition are the result of a higher number of nestlings in coniferous habitat (Mänd *et al*. 2005), because the inclusion of the number of nestlings in the models did not change the results (**III**). It must also be noted that the differences in parental condition indices between habitats were only slight despite the large sample sizes. Notably, although several condition indices differed between habitats, no habitat effect on adult survival was found (**I**; but see Mänd *et al*. 2005). Moreover, in another year, no effect of habitat on female condition during the chick-rearing period was revealed (**III**). The differences between the studies may be caused by annual differences in foraging conditions, since body condition and immune parameters of birds within one population can differ considerably between years (Acquarone *et al*. 2002; Sánches-Guzmán *et al*. 2004; Kern *et al*. 2005).

3.1.5. Seasonal variation

Almost nothing is known about the changes of physiological condition indices between different breeding attempts within one year. Similarly, the seasonal changes in condition indices between breeding stages within one breeding attempt are quite poorly known in free-living birds. This information is, however, essential if we want to use condition indices in free-living birds.

Differences between breeding attempts: At an individual level, heterophile concentration and H/L ratio were lower on average during the second than during the first breeding attempt, although the difference was significant only in one year (**II**). At the same time, no significant change at an individual level was observed in other blood parameters – hematocrit, lymphocyte concentration,

plasma albumin, globulin and triglyceride concentrations and Alb/Glob ratio (**II**). The H/L ratio in birds is widely used as a measure of stress (Gross & Siegel 1983; Ots & Hõrak 1996; Moreno *et al*. 2002; Sanz *et al*. 2004). My results therefore suggest that parents may experience more stress when caring for the first broods compared with the second broods. The second breeding attempt is usually characterized by considerably lower breeding density (Mägi & Mänd 2004) and in temperate regions, with higher air temperature compared with the first breeding attempt. Also, brood size is generally much higher during the first breeding attempt (Mägi & Mänd 2004), and previous studies have shown that the H/L ratio in birds is positively related to measures of reproductive effort (Ots & Hõrak 1996; Moreno *et al*. 2002; Sanz *et al*. 2004). However, the result did not change when the number of fledglings was included in the model (**II**). Therefore it is unlikely that it was caused by differences in reproductive effort between breeding attempts, and other explanations are needed.

Female body mass was higher on average during the first breeding attempt compared to the second breeding attempt (**II**), suggesting that the amount of body reserves may be higher during the first broods.

In another year, it was found that both caterpillar abundance and plasma carotenoid levels of incubating females increased with time during the second breeding attempt (**IV**). Since carotenoids cannot be synthesized in the body and must be obtained with food (McGraw 2006), this result compares favorably with previous findings that lepidopteran larvae serve as a main source of dietary carotenoids for breeding great tits (Partali *et al*. 1987; Eeva *et al*. 1998). This result also indicates that food abundance actually increases during the second breeding attempt. This is in accordance with the results of Mägi (2007), who showed that the food abundance in our study area is much higher during the second breeding attempt compared with the first breeding attempt.

Differences between breeding stages: There were significant changes in several blood parameters from the incubation to the chick rearing stage. The higher values of beta globulins and lower values of Alb/Glob ratio during incubation compared to the chick rearing period (**III**) may indicate higher acute inflammatory response and the lower health state at this breeding phase (Kawai 1973; Coles 1997). There was however no significant change in gamma-globulin concentrations with breeding phase (**III**). Thus, investments into humoral immune defense did not differ between the two reproductive phases. It has previously been shown in birds that immunoglobulin production increases during egg laying, in order to facilitate passive immunoglobulin transfer to eggs (Saino *et al*. 2001; Gasparini *et al*. 2002). However, in accordance with my results, it has also been shown in barn swallows that circulating immuno-globulin concentrations drop to prelaying levels with clutch completion (Saino *et al*. 2001).

Plasma triglyceride concentrations depend strongly on food intake (Jenni-Eiermann & Jenni 1997; Cerasale & Guglielmo 2006), therefore higher triglyceride concentration during incubation may indicate females' superior nutritional status compared to that during the chick rearing phase (**III**). This is in accordance with the higher body mass observed during incubation compared to the chick rearing period (**III**). Triglyceride concentrations also increase in egg-laying birds (Vanderkist *et al.* 2000; Challenger *et al.* 2001). However, it has been shown in European starlings (*Sturnus vulgaris*) that triglyceride values decreased to pre-breeding levels with clutch completion (Challenger *et al.* 2001). In pied flycatchers (*Ficedula hypoleuca*), no differences were found in triglyceride concentrations between the incubation and chick rearing stages (Kern *et al.* 2005). There were also no differences between breeding stages in albumin concentration (**III**), an indicator strongly associated with protein nutrition (Kawai 1973; Coles 1997).

Females also had higher hematocrits during chick rearing than during incubation (**III**). Hematocrit typically declines in egg-laying birds and sometimes it does not recover upon cessation of egg production, remaining low even during incubation (reviewed in Williams *et al.* 2004). However, it has also been shown in great tits that experimental brood increment increases parents' hematocrit (Hõrak *et al.* 1998b). Therefore, higher hematocrit during brood rearing, compared to the incubation stage, may also be a response to the requirement of elevated oxygen-carrying capacity of the blood during increased workload.

As shown also in other studies (*e.g.*, Freed 1981; Cichoń 2001), female body mass declined significantly from the incubation to the chick-rearing phase (**III**). However, this decline was more pronounced in coniferous habitat, where females lost ca. 0.5 g more mass compared to deciduous habitat (**III**). This could indicate, as already suggested earlier, that possible favorable conditions in coniferous habitat early in the season are no longer so prevalent in the chick rearing stage. However, the body mass decline in breeding females has been suggested to be partly a strategic adjustment in order to maintain better maneuverability needed for intensive nestling feeding (Freed 1981). Thus, it is also possible that, during the chick rearing period, female body mass was adjusted to the same level in both habitats irrespective of the habitat quality.

3.1.6. Antioxidant protection and health

Under the hypothesis that carotenoids significantly contribute to antioxidative protection, it was predicted that individual plasma carotenoid levels correlate positively with measures of total antioxidant capacity. Yet no such correlations emerged (**IV**). In line with this result, serum carotenoid concentration did not correlate with measures of antioxidant protection and serum concentration of reactive oxygen metabolites, which are a marker of early oxidative damage in a

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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 antioxidant barrier (Alonso-Alvarez *et al.* 2004b). Altogether, these findings suggest that antioxidant properties of carotenoids in birds (except well-established protective effects on embryos and hatchlings) may not be as important as previously thought, at least in situations where redox homeostasis is not threatened (Hartley & Kennedy 2004; but see Hõrak *et al.* 2007). This view would be also compatible with the results of an extensive meta-analysis of clinical studies of oxidative stress (Dotan *et al.* 2004), revealing that most antioxidants exhibit a non-monotonic relationship with other criteria of oxidative stress, so that only under severe pathological conditions do all the indices of oxidative stress correlate with each other. It is however also possible, as recently suggested by Hõrak *et al.* (2007), that measuring the antioxidant capacity (such as AOP and TAS) alone may be insufficient to reveal the antioxidant function of carotenoids.

Since oxidative stress is involved in pathogenesis of possible infectious and inflammatory disorders (Beckman & Ames 1998; Halliwell & Gutteridge 1999), a negative correlation between the indices of total antioxidant capacity and leukocytic markers of inflammation was predicted. Such a relationship, although not linear, was indeed found between both measurements of antioxidant protection, and heterophile concentration and H/L ratio (**IV**). Heterophils are inflammatory cells that bind microbes, internalize them and subsequently kill them by oxidative burst, releasing reactive oxygen and nitrogen intermediates and katabolic enzymes (Ames *et al.* 1993; Kogut *et al.* 2002). However, the same free radicals may also cause tissue damage if scavenged insufficiently (*e.g.*, Terrón *et al.* 2003). It is thus possible that the initial decrease in total antioxidant capacity with increasing heterophile concentration reflects a diminishing level of antioxidant protection, induced by inflammatory processes that are accompanied by increased traffic of heterophils in the blood stream and excess production of reactive oxygen species (Costantini & Dell'Omo 2006). In the current study, however, plasma total antioxidant capacity started to increase again at very high heterophile concentrations. One cannot therefore exclude the possibility that 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.*, Shing *et al.* 2007).

3.1.7. Conclusions

In conclusion, my results suggest that leukocytic immune parameters and plasma protein profiles can be used to predict survival in adult birds. However there was no indication that this effect on survival is caused by reproductive effort. In addition, there appeared to be no connections between condition indices and measures of reproductive effort, possibly because of the correlational nature of my results. The parents' decision to start a second clutch seems to be unrelated to their physiological condition measured at the end of the first breeding attempt. Females appeared to be more stressed and in worse health state during both breeding attempts compared to males, suggesting that brood rearing requires greater effort on the part of females. Blood parameters also appeared to be useful indicators of habitat quality. Although, during first breeding attempt, females preferred deciduous habitat fragments and laid larger clutches there, the nutritional condition during incubation was better in non-preferred coniferous habitat. In some years parents also appeared to be in slightly better health in coniferous habitat during chick-rearing time. Although it seems that habitat related differences in condition are not directly caused by habitat-specific reproductive decisions, the exact causes remain open. My results suggest that blood parameters can also be useful in future conservational studies aiming to determine the health status of threatened populations. When using blood parameters in free-living birds, individual changes in time must be taken into account. I found that there were no changes at an individual level in most blood parameters between the two breeding attempts, except for heterophile concentrations and H/L ratio. However, differences in condition indices between incubation and chick-rearing stages were remarkable, possibly as a result of a combination of causes. Although incubating females seem to be in a better nutritional state, their general health may be worse compared to chick rearing females. My results also show that there is no correlation between plasma carotenoid level and plasma antioxidant capacity. Also, the relation of antioxidant protection to leukocytic markers of inflammation indicates that antioxidant capacity deserves further attention in ecophysiological studies as potential indicators of immunopathology.

3.2. Bone alkaline phosphatase as a measure of skeletal development in great tit nestlings

3.2.1. Changes in alkaline phosphatase levels in relation to skeletal mineralization

It has remained unclear whether ALP activity in birds is related to bone mineralization that leads to the replacement of initial cartilage with minerals (mainly calcium), or to bone elongation determining the longitudinal size of the bones. To tease apart these processes, the residuals of total skeletal calcium against the weight of bones were calculated. That should reflect the mineralization process only – that is the increase in the calcium concentration.

It appeared that both bone-ALP and liver-ALP activity were positively related to the calcium concentration in the entire skeleton when chick age was controlled for (**V**). Thus it was demonstrated for the first time that the rate of bone mineralization in birds is directly related to ALP activity. Interestingly, both ALP isoforms were related to bone mineralization, although the relationship with bone-ALP was stronger than that with liver-ALP (**V**). These results are consistent with results found in humans (*Homo sapiens*) and horses (*Equus caballus*), where bone-ALP was more highly correlated with bone turnover than total-ALP consisting of both bone and liver isoforms (de Behr *et al*. 2003; Baskin *et al*. 2004). Bone-ALP contributes to bone mineralization either through the liberation of inorganic phosphate forming hydroxyapatite crystals in the bone matrix, or through the hydrolysis of pyrophosphate, the latter being a strong inhibitor of crystal formation (de Behr *et al*. 2003). Why bone mineralization was related to the activity of liver-ALP activity remains unclear. It is possible that the rate of skeletal ossification correlates with other physiological processes such as a high cellular turnover of other tissues (Alvaro *et al*. 2000; Watkins *et al*. 2000). However, bone-ALP seems to be a more sensitive marker of bone mineralization than are liver-ALP or total-ALP, and is certainly the preferred indicator.

When different body parts were considered separately, it appeared that bone-ALP was positively related to calcium concentration in leg and wing bones, whereas no association with calcium concentration in the skull was found (**V**). This may be because the skull as a flat bone shows a different type of bone formation (periosteal ossification) than the long bones (endochondral ossification; Montes *et al*. 2005). However, bone-ALP is involved in both types of bone formation and plasma bone-ALP levels should reflect the rate of mineralization of the entire skeleton. It is possible that rapid mineralization of long bones contributes relatively more to the plasmatic level of bone-ALP than delayed mineralization of flat bones such as the skull (Dobado-Berrios & Ferrer

1997; Dutta *et al*. 1998). Liver-ALP showed no significant relationships with Ca concentration either in legs, wings or skull (**V**).

Given that mineralization and elongation of long bones in chicks are closely linked processes (*e.g.*, Cubo *et al*. 2000), it was also hypothesized that bone-ALP activity is related to the growth of morphological traits as well. To estimate the relative contribution of the elongation of long bones (tarsus and wing) to ALP activity, both the length and calcium concentration of a particular limb adjusted for age were considered in the same model. However, no evidence was found that elongation of long bones is correlated with bone-ALP activity (**V**). It is possible that there might be a time lag between the elongation and the mineralization processes (Karsenty 1998; de Behr *et al*. 2003), since tarsus length had stopped growing several days before the last measurements were taken (**V**). For wings, the result may also be confounded by the fact that wing length included both skeletal and feather components.

3.2.2. Changes in alkaline phosphatase levels in relation to chick age

As chicks grow, the total amount of Ca in the skeleton as well as the Ca concentration of the skeleton increases with age (**V**). However, since the peak growth period in great tits is in the middle of the nestling period (Tilgar & Mänd 2006), one would expect the bone-ALP levels also be higher in the middle of the nestling period, compared to the pre-fledging stage. Indeed, bone-ALP activity declined significantly from the 8th to 15th day post-hatch, indicating the decelerating phase of ossification in full-gown fledglings (**VI**). There are no studies in other passerines to compare this result with. However, in another study made in the same study area but in a marginal coniferous habitat (small forest patches around urban area), there appeared to be no significant age related changes in great tit bone-ALP levels (**V**). It is possible that nestlings in those relatively poor conditions had postponed the decline of bone-ALP nearer the end of the nestling phase because of their reduced growth rates (Tilgar *et al*. 2004). It has previously been found that the dynamics of ALP throughout the growth period can be different in birds with large body size. For example, in the white stork (*Ciconia ciconia*), bone-ALP level tended to reach a maximum level shortly before fledging (Smits *et al*. 2007). Hence, it is possible that intensive ossification overlaps with the active phase of somatic growth in small birds, while these two processes appear to be temporally more separated in large birds.

It is also important to note that liver-ALP showed no significant age related dynamics in great tit chicks (**V**, **VI**). This suggests again that liver-ALP cannot be used as an indicator of chick skeletal maturity.

3.2.3. Bone alkaline phosphatase levels reflect growth conditions and chick pre-fledging maturity

As daily-ingested calcium is essential for skeletal growth in nestling passerines (*e.g.*, Barclay 1994; Bureš & Weidinger 2000; Poulin & Brigham 2001), one would expect a connection between calcium availability and plasmatic bone-ALP levels. To study the application of ALP activity as a measure of calcium availability in nestlings diet, experimental nests were supplemented with extra calcium during the whole nestling period. In one of the two study years it was found that in the calcium-supplemented group, the bone-ALP level was significantly lower in 15-day-old nestlings compared to controls (**VII**). There were no differences in total-ALP or liver-ALP between treatments (**VII**). Graveland & van Gijzen (1994) have shown that 14-day-old nestling great tits had nearly achieved their full skeletal size while their bones still contained markedly less calcium compared with those of adults. Also bone-ALP levels decline towards the end of the nestling period (**VI**). Thus, it seems that nestlings in calcium-provisioned nests had completed their skeletal growth earlier than those in control nests, and that the level of bone-ALP in their blood was declining.

However, no differences between treatments were found in the second study year (**VII**). In this year there were very unfavorable breeding conditions and probably neither calcium supplemented nor control chicks had completed their skeletal development by day 15 post-hatch. As a result, the bone-ALP of the nestlings of both treatment groups remained high. Thus bone-ALP levels should be interpreted with caution under very unsuitable climatic conditions.

It is important to note that there were no differences in morphological traits of chicks (tarsus length and body mass) between experimental treatments (**VII**). This is consistent with earlier findings in the same great tit population, that calcium supplementation causes only subtle increases in the tarsus length of fledglings (Tilgar *et al.* 2002). Thus one cannot discriminate between nestlings of different developmental stages on the basis of morphological traits.

It was previously revealed that bone-ALP activity measured shortly before fledging could reveal subtle developmental differences between nestlings of similar size (**VII**, Tilgar *et al.* 2004). It was proposed that the young with higher growth rates might complete the rapid phase of ossification earlier than those with retarded growth, and respective differences between developmental stages can be revealed by different values of bone-ALP measured at pre-fledging stage. Given that broods with higher growth rates usually fledge earlier than those with slower growth rates (Keller & van Noordwijk 1994; Viñuela & Ferrer 1997), the duration of the nestling period should be closely linked with the pre-fledging maturity of nestlings. However it has remained unclear whether bone-ALP values can be used to predict the day on which the nestlings actually leave the nest.

Paper **VI** demonstrated that bone-ALP level measured at day 15 post-hatch was positively correlated with the duration of the nestling period. There was no association between the duration of the nestling period and liver-ALP (**VI**). A prolonged nestling period extends the time period during which the young are more vulnerable to predators (Perrins 1979) and parasites (Møller 1990; Gebhardt-Heinrich & Richner 1998). Furthermore, a prolonged nestling period may decrease the fitness of the parents, because great tit females may be constrained temporally from laying second clutches in the same season (Smith *et al*. 1987; Lindén 1988).

Bone-ALP at day 15 post-hatch was inversely related to pre-fledging values of wing length and body mass, but not to tarsus length (**VI**). Morphological traits – body mass, tarsus length and wing length – were negatively but only marginally related to the duration of the nestling period (**VI**). Thus, bone-ALP seems to be a more reliable indicator of nestling maturity at the pre-fledging stage than body mass or structural measurements.

3.2.4. Conclusions

In conclusion, bone-ALP seems to be a sensitive marker of bone mineralization in passerine birds. Its values change with nestling age and in great tits tends to decrease at the end of the nestling period. This indicates a decelerating phase of ossification in full-grown fledglings. Measuring bone-ALP also offers a complementary bioassay to reveal subtle developmental differences between chicks nurtured in environments differing in calcium availability. As calcium metabolism and bone formation can be disturbed by various environmental contaminants (Eeva & Lehikoinen 1996; Smits *et al*. 2007), bone-ALP has also the potential to be useful indicator in conservation biology (Smits *et al*. 2007). Bone-ALP also seems to be a better indicator of nestlings pre-fledging maturity than liver- or total-ALP or morphological measurements. Furthermore, measuring bone-ALP at the end of the nestling period allows the prediction of the duration of the nestling period. Thus, bone-ALP values in nestlings can potentially be related to the subsequent fitness of nestlings and parents. Since there has been shown to exist a trade-off between growth and immune system (Soler *et al*. 2003; Brommer *et al*. 2004), bone-ALP can potentially also be used in future studies looking at trade-offs between skeletal development and other functions of the body. However, interpretation of bone-ALP variation in free-living birds must be made with caution, because growth and bone-ALP levels are affected by environmental conditions during growth.

SUMMARY

To explain the physiological trade-offs underlying the life-history variation in birds, we need to measure individual physiological state in the wild. Similarly, in conservation biology, the assessment of individual physiological status in the wild is often the only way to measure the well-being of individuals and populations in a particular environment. The general aim of the thesis was to describe the natural causes of variation in several blood parameters in adult and nestling great tits (*Parus major*) and to apply them in the studies of the reproductive ecology of birds. In the first part of the present thesis I examine the condition indices in breeding adult great tits. In the second part of the thesis I examine the variation of the level of alkaline phosphatase in the plasma of great tit nestlings.

To interpret the changes in condition indices of free-living birds, it is necessary to know how they are related to fitness components such as survival and reproduction. It was found that surviving adults had higher albumin/ globulin ratio, lower plasma globulin concentration and lower heterophile/ lymphocyte ratio during the breeding period in the preceding year, compared to non-survivors. Surviving males also had more lymphocytes compared to non-survivors. Thus, better immunological status and lower stress was associated with higher survival. However, there was no indication that this effect on survival is caused by reproductive effort. In addition, no connection appeared to exist between condition indices and measures of reproductive effort, possibly because of the correlational nature of the results. Also the parents' decision to start a second clutch was unrelated to their physiological condition measured at the end of the first breeding attempt.

Chick rearing female great tits had lower size-corrected body masses, more heterophiles, a higher H/L ratio, higher globulin concentration and a lower albumin/globulin ratio, and during the first breeding attempt, also higher hematocrit than males. Thus females appeared to be more stressed and in a worse health state during both breeding attempts compared to males, suggesting that brood rearing has a greater impact on females.

Blood parameters appeared to be useful as indicators of habitat quality. Females preferred deciduous habitat fragments and laid larger clutches there compared to coniferous habitat. However, unexpectedly, while incubating their first clutches, females in coniferous habitat had higher size-corrected body mass, hematocrit, plasma albumin/globulin ratio and albumin concentration compared to those in deciduous habitat. Thus, the nutritional condition of incubating females during the first breeding attempt was better in non-preferred coniferous habitat than in preferred deciduous habitat. In accordance with pre-vious findings from the same population that the final breeding success is also often better in coniferous habitat, I found that in some years parents also appeared to be in slightly better health in coniferous habitat during the chick-

rearing period. The reasons for the above mentioned habitat related differences in adults' condition remain open. However, it seems that they are not directly caused by habitat-specific reproductive decisions.

I found that there were no changes at an individual level in condition indices between two breeding attempts, except for body mass, heterophile concentrations and heterophile/lymphocyte ratio. However, the differences in several condition indices between incubation and chick-rearing stages were remarkable and complex, possibly as a result of a combination of causes. In addition, seasonal changes in caterpillar abundance in the environment were reflected in concordant changes in female plasma carotenoids. Thus, when using blood parameters in free-living birds, individual changes in time must be taken into account.

No correlation was detected between plasma carotenoid levels and measures of total antioxidant capacity. However, a strong non-linear association between the measures of antioxidant protection and heterophile concentration suggests that measures of total antioxidant capacity deserve further attention in eco-physiological studies as potential indicators of immunopathology.

This thesis suggests that bone alkaline phosphatase (bone-ALP) can be used as a sensitive indicator of the rate of bone mineralization in birds. This is because the activity of bone-ALP was closely related to the overall rate of skeletal mineralization in great tit nestlings. In addition, bone-ALP activity predicted the rate of mineralization of leg and wing bones, but not that of the skull. Bone-ALP values also changed with nestling age and tended to decrease at the end of the nestling phase, suggesting a decelerating phase of ossification in full-grown nestlings. It was also experimentally confirmed that bone-ALP levels are affected by the amount of calcium in a nestling's diet, thus reflecting the nestling's growth conditions and maturity. Furthermore, bone-ALP levels measured at the end of the nestling stage predicted the actual day of fledging. Bone-ALP also seemed to be a better indicator of nestlings' pre-fledging maturity than liver-ALP, total ALP or morphological measurements. Thus, bone-ALP levels in nestlings can potentially be related to nestlings' and parents' subsequent fitness. However, interpretation of bone-ALP variation in free-living birds must be made with caution, because the growth and bone-ALP levels are affected by environmental conditions during growth.

In conclusion, I believe that the results of this thesis improve our understanding about the causes of variation in blood parameters of condition and development. Such knowledge can be useful in future studies in the fields of ecological, conservational, physiological and evolutionary research.

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KOKKUVÕTE

Vereparameetrid kui füsioloogilise konditsiooni ja luustiku arengu näitajad rasvatihasel (*Parus major*): looduslik varieeruvus ja rakendamine lindude sigimisökoloogias

Selleks, et seletada elukäikude varieeruvuse aluseks olevaid lõivsuhteid lindudel, on tihti vaja mõõta isendite füsioloogilist seisundit looduses. Samuti on looduskaitsebioloogias isendite füsioloogilise seisundi mõõtmine sageli ainus viis, kuidas hinnata isendite ja populatsioonide seisundit nende elukeskkonnas. Käesoleva dissertatsiooni eesmärgiks oli uurida mitmete verest mõõdetavate füsioloogiliste parameetrite looduslikku varieeruvust rasvatihasel (*Parus major*) ja rakendada neid sigimisbioloogilistes uuringutes. Dissertatsiooni esimeses pooles käsitlen pesitsevate rasvatihase vanalindude konditsiooninäitajaid. Dissertatsiooni teises pooles uurin rasvatihase poegade vereplasma aluselise fosfataasi kasutamist luustumise indikaatorina.

Selleks, et interpreteerida konditsiooninäitajates toimuvaid muutusi vabalt elavatel lindudel, on vaja teada, kuidas konditsiooninäitajad on seotud kohasuse komponentidega – elumuse ja sigimisega. Leidsin et vanalindude pesitsuse-aegne immunoloogiline seisund oli seotud nende edasise ellujäämusega. Ellu-jäänud lindude vereplasmas oli eelmisel aastal oluliselt kõrgem albumiini/ globuliini suhe, madalam globuliinide kontsentratsioon ja madalam hetero-fiilide/lümfotsüütide suhe, võrreldes nende lindudega, kes järgmise aastani ei elanud. Ellujäänud isaslindudel oli ka kõrgem lümfotsüütide kontsentratsioon, võrreldes mitte-ellujäänutega. Ei leitud tõendeid, et see mõju ellujäämusele oleks olnud põhjustatud sigimispingutuse poolt. Samuti ei leitud korrelatiivses analüüsis seoseid sigimispingutuse näitajate ja konditsiooniindeksite vahel. Ka vanalindude otsus, kas pesitseda sessooni jooksul veel teist korda või mitte, ei sõltunud nende konditsiooninäitajatest esimese pesitsuskorra lõpus.

Selgus, et emastel lindudel oli poegade kasvatamise perioodil oluliselt mada-lam suhteline mass, kõrgem heterofiilide kontsentratsioon, kõrgem heterofiilide/ lümfotsüütide suhe, kõrgem globuliinide kontsentratsioon, madalam albumiini/ globuliini suhe ja esimesel pesitsuskorral ka kõrgem hematokrit kui isastel. Seega paistavad emased linnud olevat nii esimesel kui ka teisel pesitsuskorral poegade kasvatamise ajal isastega võrreldes rohkem stressis ja kehvemas tervis-likus seisundis.

Verenäitajad osutusid ka kasulikuks elupaiga kvaliteedi indikaatoriks. Ema-sed rasvatihased eelistasid pesitseda lehtmetsa fragmentides ja munesid seal suuremaid kurni kui okasmetas. Üllataval kombel oli nende esimese pesitsuse aegne toitumuslik seisund haudumise perioodil aga parem hoopiski okasmetsas. Okasmetsas oli emaslindudel kõrgem suhteline mass, hematokrit, plasma albumiini/globuliini suhe ja albumiini kontsentratsioon võrreldes lehtmetsa lindudega. Kooskõlas varasemate tulemustega samast populatsioonist, et ka

sigimiseedukus on parem okasmetsas leidsin et okasmetsa linnud olid mõnevõrra paremas tervislikus seisundis ka poegade toitmise ajal. Eelpool mainitud elupaikade vaheliste erinevuste täpsed põhjused jäid lahtiseks. Samas ilmnes, et need ei olnud otseselt seotud elupaiguti erinevate sigimisotsustega.

Sama sesooni kahe järjestikuse pesitsuskorra võrreldavates faasides ei täheldatud enamikus konditsiooninäitajates olulisi muutusi, välja arvatud kehamassis, heterofiilide kontsentratsioonis ja heterofiilide/lümfotsüütide suhtes. Paljud konditsiooninäitajad muutusid aga oluliselt ühe ja sama pesitsuskorra jooksul haudumisest kuni poegade toitmise ajani. Samuti peegeldus ajaline muutus röövikute arvukuses emaslindude plasma karotenoidide kontsentratsioonis. Seetõttu tuleks konditsiooniindeksite kasutamisel vabalt elavatel lindudel kindlasti arvesse võtta nende indeksite ajalist muutlikkust.

Käesolevas töös ei leitud olulist seost plasma karotenoidide kontsentratsiooni ja üldise antioksüdatiivse potentsiaali vahel. Samas leiti tugev mittelineaarne seos üldise antioksüdatiivse potentsiaali ja heterofiilide kontsentratsiooni vahel, mis näitab, et antioksüdatiivse potentsiaali mõõtmine väärib edasist tähelepanu kui oluline immunopatoloogia näitaja.

Selles töös leiti ka, et luu aluselise fosfataasi (luu-*ALP*) tase vereplasmas võib olla kasutatav luustumise tundliku indikaatorina lindudel, kuna tema väärtused on tihedalt seotud skeleti üldise luustumisega rasvatihase poegadel. Luu-*ALP* peegeldas ka eraldi jala- ja tiivaluude luustumist, kuid mitte kolju luustumist. Luu-*ALP*'i väärtused muutusid ka poegade vanusega ja kaldusid enne lennuvõimestumist langema. See osundab, et morfoloogiliselt lõppsuuruse saavutanud poegade luustumine oli aeglustunud. Samuti näidati eksperimentaalselt, et luu-*ALP*'i väärtused on mõjustatud kaltsiumi hulga poolt poegade toidus. Seega peegeldavad luu-*ALP*'i väärtused omakorda poegade kasvukeskkonna tingimusi. Veelgi enam, lennuvõimestumiseelsed luu-*ALP*'i väärtused ennustasid pesakonna lennuvõimestumise tegelikku päeva usaldusväärsemalt kui morfoloogilised näitajad. Samuti oli luu-*ALP* parem poegade lennuvõimestumiseelse küpsuse näitaja kui maksa-*ALP* või üldine aluselise fosfataasi tase vereplasmas. Nende tulemuste taustal on võimalik, et luu-*ALP* võimaldab ennustada ka poegade ja vanalindude edasist kohasust. Kuid luu-*ALP*'i väärtuste varieeruvuse interpreteerimisse looduses elavatel lindudel tuleb suhtuda ettevaatusega, kuna nii kasv kui ka luu-*ALP*'i väärtused sõltuvad kasvuperioodil valitsenud keskkonna tingimustest.

Kokkuvõttes arvan, et käesoleva dissertatsiooni tulemused aitavad parandada meie teadmisi lindude konditsiooni ja kasvuga seotud vereparameetrite varieeruvuse põhjustest ja seaduspärasustest. Nendest teadmistest võib omakorda kasu olla tulevastes ökoloogilistes, füsioloogilistes, looduskaitselistes ja evolutsioonilistes uuringutes.

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PUBLICATIONS

I

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II

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Hematological parameters in brood-rearing great tits in relation to habitat,
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III

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Physiological condition of incubating and brood rearing
female Great Tits *Parus major* in two contrasting habitats.
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Physiological condition of incubating and brood rearing female Great Tits *Parus major* in two contrasting habitats

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ABSTRACT

Little is known about the physiological causes and consequences of habitat choice decisions in birds. We compared size-corrected body mass, hematocrit, plasma albumin, beta-, and gamma-globulin and triglyceride concentrations, and albumin/globulin ratio of female Great Tits breeding in deciduous and coniferous forests in Estonia. Females were sampled during incubation and during the chick-rearing period. While the nest box occupation rate and clutch size were higher in the deciduous habitat, the female nutritional condition during incubation was better in the coniferous habitat. This habitat difference in condition was not explained by the differences in the start of egg-laying or clutch size. Females lost more mass from incubation to chick rearing stage in the coniferous than in the deciduous forests. Although the values of most blood parameters changed from incubation to chick rearing stage, no habitat related change was observed. There were no habitat differences in the number and quality of fledglings or in female condition during chick rearing period. Our results indicate that incubating female great tits are in worse nutritional state in the preferred deciduous habitat fragments compared to those in the non-preferred coniferous habitat. However, habitat-related differences in condition during incubation seem to be unrelated to habitat-specific reproductive decisions.

Key words: Great Tit, *Parus major*, habitat differences, plasma proteins, triglycerides, hematocrit, breeding cycle

Running title: Habitat differences in physiological condition

INTRODUCTION

Due to the loss and fragmentation of natural forest habitats, the ecological quality of the remaining plots changes (reviewed in Ewers & Didham 2006). With such decrease of the amount of optimal habitat, increased proportion of individuals is often forced to move to normally avoided or non-preferred habitats (Battin 2004). Sometimes birds are attracted to non-preferred habitats also by conservationists and researchers who use some wildlife management techniques, e.g., providing nest boxes for secondary cavity-nesting birds (Rodenhouse et al.1997, Semel & Sherman 2001, Schlaepfer et al. 2002). However, our knowledge about the individual physiological causes and consequences of habitat choice decisions in such mosaics of optimal and sub-optimal habitat is still very limited (e.g. Mazerolle & Hobson 2002, Suorsa et al. 2003, 2004, Wikelski & Cooke 2006, Bańbura et al. 2007, Johnson 2007).

Several hole-nesting passerines, including the Great Tits *Parus major* prefer to breed in deciduous woodlands instead of coniferous forests (van Balen 1973, Klomp 1980, Mänd et al. 2005, reviewed in Newton 1998). Coniferous forests are considered to be less attractive for breeding as a result of nest hole shortage and lower food abundance (van Balen 1982, Perrins 1991). Also the breeding success of Great Tit has generally been shown to be higher in deciduous habitats compared to coniferous habitat (Sanz 1998; but see Lundberg et al. 1981, Catalan & Haeger 1996, Mänd et al. 2005).

In our study area, situated in north-eastern Europe, it has recently been shown that the occupancy of the nest boxes by Great Tits is higher and clutch and egg sizes are larger in the fragments of deciduous habitat compared to managed pine forests (Mänd et al. 2005). Unexpectedly, the number of offspring and their condition turned out to be higher in coniferous habitat in most years (Mänd et al. 2005). Furthermore, adults' physiological condition during nestling feeding period tended to be better in the coniferous habitat compared to the deciduous habitat (Kilgas et al. 2006). However, it has remained unsolved whether such habitat differences in adult condition arise during nestling feeding period or already in earlier breeding phases.

The decrease of body mass during breeding is a commonly observed phenomenon in female birds (e.g. Freed 1981, Merilä & Wiggins 1997, Cichoń 2001). Much less is known about changes of physiological parameters throughout the different reproductive phases in free-living birds (e.g. Hõrak et al. 1998a, Vanderkist et al. 2000, Kern et al. 2005, Gayathry & Hedge 2006). However, to our knowledge, the studies examining temporal changes in physiological condition indices of breeding birds simultaneously in different habitats are absent.

In the current study we compared the nutritional and immunological condition and body size of breeding female Great Tits in deciduous and coniferous forests of a heterogeneous habitat mosaic. Blood samples were taken

in the middle of incubation period and in the second half of the nestling period. We also recorded breeding data (see Material and Methods) to see whether habitat-related differences in physiological condition are also in correspondence with the habitat-specific reproductive decisions. We also followed individual changes in condition indices from incubation to chick rearing stage in both habitats. Based on our previous results (see above), it was predicted that female condition during incubation is better in preferred deciduous habitat than in non-preferred coniferous habitat. The opposite was predicted for chick rearing period. As indicators of female physiological condition, we used size-corrected body mass, hematocrit, plasma albumin, beta- and gamma-globulin and triglyceride concentrations, and albumin/globulin ratio.

MATERIAL AND METHODS

General field procedures

Data were collected in the surroundings of Kilingi-Nõmme in Estonia (58º 7' N, 25º 5' E), NE Europe, in 2003. The study area is approximately 50 km^2 with two contrasting forest types - deciduous and coniferous (see, Mänd et al. 2005 for the scheme of the study area). Fragments of young deciduous forests, surrounded by agricultural landscape, grow on fertile soils that have a rich deciduous understorey. The most common tree species are Grey Alder *Alnus incana* and Silver Birch *Betula pendula*. The coniferous habitat is a typical continuous managed pine forest on nutrient-poor sandy or peat (in the lower parts of the terrain) soil. Dominant tree species are Scots Pine *Pinus sylvestris*, which sometimes occur in mixed stands of Norway Spruce *Picea abies* on sandy soils or of Downy Birch *Betula pubescens* on peat soils.

Great Tits bred in wooden nest boxes with cavity measurements of 11×11×30 cm and an entrance diameter of 3.5-4.0 cm. Nest boxes were mounted on tree trunks at a height of 1.5-2.0 m. The nest boxes were arranged in lines, and each line consisted of some tens of nest boxes. Distance between nest boxes was 50-60 m. Old nest material was removed every year before the start of nest building. All nest boxes were visited twice (with one-week interval) during the nest-building period, in order to record those occupied by great tits. Each occupied nest box was thereafter inspected with 1-2-day intervals during the whole egg-laying period.

Incubating females were weighed on the fifth day after the clutch completion with a Pesola spring balance to the nearest 0.1 g and their tarsi were measured with a sliding calliper to the nearest 0.1 mm. Blood samples (ca 100–150 microliters) were taken from the tarsal or brachial veins and collected into heparinized capillary tubes and kept cool until centrifugation few hours later. 37% of incubating females abandoned the nest after the capture and blood

sampling. There were no differences in incubation body mass or blood parameters between deserters and non-deserters (T-test, all $p > 0.1$). Females that did not abandon their nests were sampled again when their nestlings were 8-15 days old. Physiological parameters of females were not significantly associated with the time of sampling or the age of nestlings (Pearson correlation, all $p > 0.1$). Fledglings' mass and tarsus length were measured at day 15 post-hatch.

Laboratory analyses

Plasma was separated from blood cells after 10 min centrifugation at 10,000 rpm, and was stored at –20°C until analysed. Hematocrit was measured with a sliding calliper to the nearest 0.1 mm. Standard agarose gel electrophoresis with REP System (Helena Laboratories) was used for detection of major protein groups. Gels were stained with Ponceau S stain using REP Gel processor, and were densitometrically scanned at a wavelength of 525 nm. Due to difficulties in separating the pre-albumin fraction from albumin, summed concentration was used and termed as albumin concentration, similarly to Ots et al. (1998). Concentration of triglycerides was determined using a quantitative enzymatic test (Sigma Diagnostics). Exactly the same methods for protein and triglyceride detection have also previously been used in Great Tits (Ots et al. 1998, Kilgas et al. 2006, Tummeleht et al 2006).

Blood parameters studied

Hematocrit measures the relative amount of red blood cells in total blood volume. It reflects the oxygen carrying capacity of the blood. Its low values (anemia) are indicative of bacterial or parasite infections, but also reflect deficiencies in nutrition, especially the scarcity of some micronutrients such as Fe, Cu and vitamin B_{12} (Svensson & Merilä 1996; Coles 1997). Hematocrit value can also rise with hard physical exercise (Saino et al. 1997) and decrease in egg laying birds (reviewed in Williams et al. 2004).

A decrease in albumin concentration in the blood plasma accompanies almost any diseases and is also an indicator of malnutrition (e.g., Kawai 1973; Ots et al. 2001, Hõrak et al 2004). The gamma-globulin fraction of the serum includes most of the known antibodies (immunoglobulins) involved in the immune response to protozoan, bacterial and viral infections. The concentration of gamma-globulins increases with both acute and chronic infections. The beta-globulins include several acute phase proteins. An increase in beta-globulin concentration in blood plasma is expected with acute inflammation (Coles 1997). Healthier individuals in general have higher albumin/globulin ratios (Kawai 1973; Coles 1997, Hõrak et al. 2004).

Triglyceride concentration in blood plasma reflects the rate of lipid transport into adipose tissues and can be considered as an estimate of the amount of lipids absorbed during the few hours before blood sampling (e.g., Jenni-Eiermann & Jenni 1997).

Statistical analyses

Statistical analyses were performed using the statistical package Statistica 7.0 (StatSoft Inc.). GLM analysis was used to study the effect of habitat on body mass, body size and blood parameters. In order to normalize the distribution of the dependent variables, original values of blood parameters, except hematocrit, were ln-transformed. To control for structural size when analysing the variation of adult body mass, the cubed tarsus length was included as a covariate in the model (García-Berthou 2001). Sample sizes differ slightly for different variables, since not all measures could be obtained for all individuals. When analyzing the effects of habitat on the number of fledglings, depredated or deserted nests were omitted from the analysis. The brood mean nestling body mass and tarsus length were used in analyses.

RESULTS

The nest box occupation rate by great tits was higher in deciduous (23.4 % of 380 nest boxes) than in coniferous (10.8 % of 776 nest boxes) habitat (χ^2 analysis, $\chi^2 = 31.8$, df = 1, p < 0.001). The great tit females breeding in deciduous forests laid slightly larger clutches than those breeding in coniferous forests (deciduous: mean $11.43 \pm$ s.d. 1.27; coniferous: 10.81 ± 1.26; GLM, habitat: $F_{1, 65} = 3.99$, P = 0.050). This result, as well as all the following results, was unchanged when laying initiation date was included in the models as a covariate. However, laying initiation date itself remained always non-significant and was therefore not included in final models. The mean date of laying start also did not differ between habitats (T-test, $t_{1, 65} = 0.13$, P = 0.9).

During incubation, females breeding in coniferous habitat had higher size-corrected body mass, hematocrit, albumin/globulin ratio and albumin concentration, compared to those breeding in deciduous habitat (GLM, Table 1). There was no significant effect of habitat on tarsus length and globulin and triglyceride concentrations (GLM, Table 1).

The inclusion of clutch size in the models as a covariate did not change the previous results. As the effect of clutch size on condition indices was not significant (results not shown), it was not included in final models.

In case of blood parameters, the results remained unchanged when female body mass was included in the models as a covariate (results not shown). As the

effect of body mass was non-significant in all initial models, it was not included in final models (Table 1).

There was no significant effect of habitat on the number of fledglings, and their tarsus length and body mass (GLM, all p > 0.1). There were also no significant effects of habitat on female tarsus length and condition indices measured during chick rearing phase (GLM, all p > 0.1).

None of the blood parameters measured in females during incubation was correlated with respective values measured during nestling phase (Pearson correlation, all p > 0.1). However, female body mass measured during incubation was positively correlated with body mass measured during nestling phase (r = 0.66, N = 37, P < 0.001).

There was a significant decrease in female body mass from incubation to chick rearing stage (Repeated measures GLM, Table 2, Figure 1). This change was significant in both habitats separately (Tukey, deciduous habitat p < 0.001 and coniferous habitat p < 0.001), although the significant breeding stage x habitat interaction term indicated that the decrease was more pronounced in coniferous habitat (Table 2, Figure 1). Female hematocrit and albumin/globulin ratio increased, and triglyceride and beta-globulin concentrations decreased from incubation to chick rearing phase (Table 2). There was no significant change in albumin or gamma-globulin concentrations (Table 2). Changes in blood parameters between different breeding stages did not differ between habitats; therefore the habitat term was not included in the final models (Table 2).

DISCUSSION

Habitat differences of condition indices

We found that, during incubation, Great Tit females in coniferous habitat had higher size-corrected body mass, hematocrit, plasma albumin/globulin ratio and albumin concentration, compared to those breeding in deciduous habitat. These results indicate that females were in a better nutritional condition (see Material and Methods) during incubation in coniferous than in deciduous habitat. Several studies have used hematological parameters and plasma chemistry to examine habitat differences in physiological condition of adult free-living birds (e.g., Mazerolle & Hobson 2002, Suorsa et al. 2004, Owen et al. 2005, Kilgas et al. 2006, Tummeleht et al. 2006). However, almost nothing is known about possible temporal patterns of habitat differences in physiological condition (reviewed in Johnson 2007). Our finding is of particular interest in the light of numerous studies that clearly show that great tits prefer deciduous habitats to coniferous habitats (e.g. van Balen 1973, Klomp 1980, Cramp and Perrins 1993, Mänd et al. 2005, this study). The fact that females usually have less and

smaller eggs per clutch in coniferous than in deciduous habitat (Sanz 1998, Mänd et al. 2005, current study) may lead one to the assumption that the observed habitat-specific variation in female condition is just the result of a lower investment in egg production (Williams 2005) or of lower incubation costs (Hanssen et al. 2005) in coniferous habitat. However, inter-habitat differences in body mass and blood parameters of females persisted even when clutch size was included in the model as a covariate. This indicates that the effect of habitat on female condition is probably independent of the egg-laying and incubation costs, and is apparently caused by some other habitat-related factors.

We cannot exclude the possibility that the habitat-related differences in female condition existed already before egg laying. This could be possible if only females in superior condition usually settle into non-preferred coniferous habitat. However, individuals in prime condition are generally expected to settle into preferred habitats (Newton 1998, Stamps 2006).

It is also possible that foraging conditions early in the season are actually better in coniferous habitat than in deciduous habitat. In the Netherlands, it has been found that the caterpillar abundance during nestling feeding stage is higher in mature oakwoods, compared to pinewoods (van Balen 1973). However, in Finland, there seemed to be no differences between birch forests and coniferous forests in caterpillar abundance during incubation period (Rytkönen & Orell 2001). Similarly, Mägi (2007) has found no differences between habitats in caterpillar biomass during incubation period for our study area. As the breeding density of Great Tits in our study area is much higher in young deciduous habitat fragments compared to coniferous habitat (Mänd et al. 2005, this study) it is possible that the *per capita* amount of food is actually higher in coniferous habitat.

Females lost ca. 0.5 g more mass from incubation to chick rearing period in coniferous compared to deciduous habitat. This could indicate that possible favorable conditions in coniferous habitat early in season are not prevalent anymore in chick-rearing stage. Consistent with this prediction, there was also no habitat effect on breeding success or female condition during the chick-rearing period. Previous studies in the same population have also failed to observe any habitat differences in the body mass (Mänd et al. 2005, Kilgas et al. 2006) or tarsus length (Mänd et al. 2005) of chick rearing females. However, we have shown earlier that brood-rearing adults tended to have a better immunological state in coniferous habitat compared to deciduous habitat (Kilgas et al 2006). The differences between the studies may be caused by annual differences in foraging conditions, since body condition and immune parameters of birds within one population can differ considerably between years (Acquarone et al. 2002, Sánches-Guzmán et al. 2004, Kern et al. 2005, Kilgas et al. 2006). Similarly, the habitat-related differences in reproductive performance

in great tits vary considerably yearly (Mänd et al. 2005, Pimentel & Nilsson 2007).

Variation in blood parameters in relation to stage in the breeding cycle

Although the values of most blood parameters showed remarkable changes between the two breeding phases, no habitat-specific changes could be revealed. The higher values of beta-globulins and lower values of albumin/globulin ratio during incubation, observed in this study, may indicate higher acute inflammatory response and the lower health state at this breeding phase (Kawai 1973, Coles 1997), compared to chick rearing period. There was however no significant change with breeding phase in gamma-globulin concentrations. Thus investments into humoral immune defence did not differ between the two reproductive phases. It has previously been shown in birds that immunoglobulin production increases during egg laying, in order to facilitate passive immunoglobulin transfer to eggs (Saino et al. 2001, Gasparini et al. 2002). However, it has also been shown in Barn Swallows *Hirundo rustica* that circulating immunoglobulin concentrations drop to prelaying levels with clutch completion (Saino et al. 2001).

Plasma triglyceride concentrations depend strongly on food intake (Jenni-Eiermann & Jenni 1997, Cerasale & Guglielmo 2006), therefore higher triglyceride concentration during incubation may indicate females' superior nutritional status compared to that during chick rearing phase. This is in accordance with higher body mass observed during incubation compared to chick rearing period. Triglyceride concentrations also increase in egg-laying birds (Vanderkist et al. 2000, Challenger et al. 2001). However, it has been shown in the European starlings *Sturnus vulgaris* that triglyceride values decreased to pre-breeding levels with clutch completion (Challenger et al. 2001). In Pied Flycatchers *Ficedula hypoleuca*, no differences were found in triglyceride concentrations between incubation and chick rearing stages (Kern et al. 2005). We also found no differences between breeding stages in albumin concentration, an indicator strongly associated with protein nutrition (Kawai 1973, Coles 1997).

Females had also higher hematocrits during chick rearing than during incubation. Hematocrit typically declines in egg-laying birds and sometimes it does not recover upon cessation of egg production, remaining low even at incubation (reviewed in Williams et al. 2004). However, it has also been shown in great tits that experimental brood increment increases parents' hematocrit (Hõrak et al. 1998b). Therefore, higher hematocrit during brood rearing, compared to incubation stage, may also be a response to the requirement of elevated oxygen-carrying capacity of the blood during increased workload.

In conclusion, our results indicate that, although great tits prefer deciduous habitat to coniferous habitat, the nutritional condition of incubating females was better in the non-preferred coniferous habitat in our study area. The decline of female body mass from incubation to chick rearing phase was more pronounced in coniferous habitat compared to deciduous habitat. Although the values of most blood parameters changed from incubation to chick rearing stage, no habitat related change was observed. There were also no habitat differences in breeding success and female condition at chick rearing stage. These findings show that the critical period that causes the habitat-related variation in physiological condition in great tits may lie at a much earlier stage of breeding than the brood-rearing stage. This study also suggests that habitat related differences in nutritional state during incubation could be unrelated to habitat-specific reproductive decisions.

SUMMARY

Little is known about the physiological consequences of habitat choice decisions in birds. In this study we compared the physiological condition of female Great Tits *Parus major* breeding in nest boxes in the fragmented deciduous forests and managed continuous coniferous forests in Estonia. As indicators of female condition, we measured size-corrected body mass, hematocrit, plasma albumin, beta-, and gamma-globulin and triglyceride concentrations, and albumin/globulin ratio. Females were sampled in the middle of incubation and at the end of the chick-rearing period. While the nest box occupation rate and clutch size were higher in the deciduous habitat, the female nutritional condition during incubation was higher in the coniferous habitat (Table 1). This habitat difference in incubation condition was not explained by the differences in the start of egg-laying or clutch size. However, females lost more mass from incubation to chick rearing stage in the coniferous than in the deciduous forests (Figure 1). Most blood parameters showed remarkable changes from incubation to chick rearing stage, however no habitat-specific changes could be detected (Table 2). There were also no habitat differences in the number and quality of fledglings or in female condition during chick rearing period. Our results indicate that incubating female great tits are in a worse nutritional state in the preferred deciduous habitat fragments compared to those in the non-preferred coniferous habitat. However, habitat-related differences in nutritional state during incubation seem to be unrelated to habitat-specific reproductive decisions.

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Table 1. The effect of habitat on condition indices and tarsus length of female Great Tits measured during incubation (GLM analysis). Presented are untransformed means. In case of body mass, the cubed tarsus length of females was included in the model as a covariate ($F_{1, 64} = 3.85$, $p = 0.05$).

	Coniferous forest			Deciduous forest				
	Mean	SD	N	Mean	SD	N	F	p
Tarsus (mm)	19.73	0.53	32	19.77	0.51	36	0.12	0.73
Mass (g)	20.59	0.79	32	20.02	0.89	35	8.28	0.005
Hematocrit	0.46	0.03	31	0.44	0.03	33	7.26	0.009
Albumin (g/L)	21.79	8.31	27	16.33	5.06	32	10.42	0.002
Beta-globulin (g/L)	7.23	2.39	27	6.39	1.53	32	2.23	0.14
Gamma-globulin (g/L)	2.89	1.18	27	2.78	0.99	32	0.16	0.69
Albumin/globulin ratio	1.47	0.34	27	1.19	0.30	32	11.57	0.001
Triglycerides (mg/dL)	252.4	142.9	27	221.5	82.6	32	0.15	0.70

Table 2. Changes of condition indices from incubation stage to chick rearing stage in female Great Tits (Repeated measures GLM). Presented are untransformed mean values. The values are pooled over habitat since it did not have significant interactions with breeding stage. In case of body mass, the term time x habitat was included in the model ($F_{1, 35} = 7.53$, $p = 0.009$).

	Incubation		Chick rearing				
	Mean	SD	Mean	SD	N	F	p
Mass (g)	20.4	0.91	18.0	0.90	38	411.8	<0.0001
Hematocrit	0.45	0.03	0.50	0.04	35	67.3	<0.0001
Albumin (g/L)	18.64	7.13	18.14	3.06	24	0.04	0.85
Beta-globulin (g/L)	7.16	1.87	5.63	1.44	24	14.9	<0.001
Gamma-globulin (g/L)	2.85	1.07	2.41	0.89	24	3.11	0.09
Albumin/globulin ratio	1.26	0.34	1.56	0.28	24	16.3	<0.001
Triglycerides (mg/dL)	244.9	126.4	153.4	68.5	26	13.9	<0.001
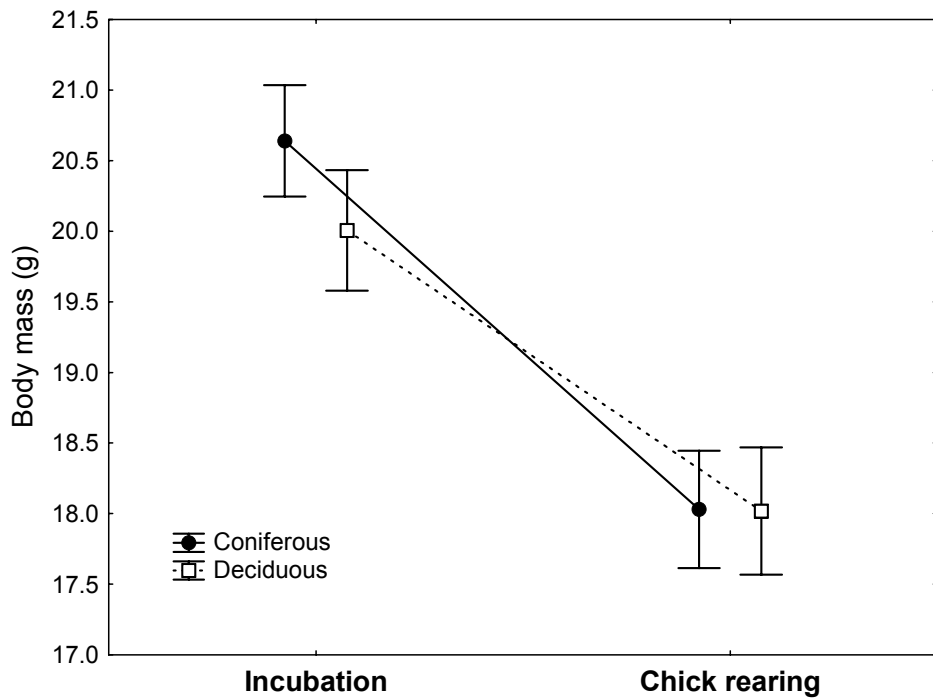
Figure 1. Body mass (mean ± 95%) of female Great Tits during incubation and during chick rearing in coniferous and deciduous habitat. Filled symbols: coniferous habitat, empty symbols: deciduous habitat.

IV

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Age-related changes in the activity of bone alkaline phosphatase and its application as a marker of pre-fledging maturity of nestlings in wild passerines

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ABSTRACT

Recent studies have suggested that bone alkaline phosphatase (bone-ALP) may be a valuable indicator of skeletal development in wild birds. However, the information about age-related dynamics of ALP isoforms in passerines is very scanty. We examined age-related changes in the activity of bone- and liver-ALP and tested the applicability of these isoenzymes as indicators of chick maturity in randomly selected nestlings of a small passerine bird, the Great Tit *Parus major*. Bone-ALP activity was elevated in the middle of the nestling period (day 8) when skeletal growth is assumed to be most rapid, and declined significantly during the pre-fledging stage (day 15 post-hatch). Bone-ALP activity at this age was positively and highly significantly related to the overall duration of nestling period and negatively and less significantly to wing length and body mass. All three morphological traits of the 15-day-old nestlings were nearly significantly and negatively correlated with the duration of the nestling period. Liver-ALP activity neither changed with nestling age, nor was related to nestling morphology. We suggest that pre-fledging activity of bone-ALP is a more reliable indicator of nestling maturity than traditionally used morphological measurements.

Key Words: alkaline phosphatase, Great Tit, *Parus major*, maturity, growth

It is widely known that newly hatched birds, with initial size differences and different growth trajectories, often achieve similar body sizes before fledging (Smith and Wettermark 1995, Gebhardt-Heinrich and Richner 1998, Badyaev and Martin 2000, Metcalfe and Monaghan 2001). However, a question remains whether catch-up growth in certain body traits (e.g., in body mass or tarsus length) reflects compensation in all developmental aspects. For example, brood

members may be indistinguishable in overall skeletal size, but may exhibit developmental differences in plumage and in soft, flexible leg bones (Tilgar et al. 2004a, b). Hence, achieving full skeletal size alone is not a reliable indication of developmental stage (Ricklefs 1975, Graveland and van Gijzen 1994, Dobado-Berrios and Ferrer 1997). Therefore, assessing nestling quality shortly before fledging merely on the basis of morphological traits may entail considerable error. One can even speculate that a depressed growth rate and delayed ossification may result in a prolonged nestling period, extending the time during which the young are vulnerable to predators (e.g., Perrins 1979) and parasites (Møller 1990, Gebhardt-Heinrich and Richner 1998). Alternatively, if the fledging date of less developed nestlings is not postponed, then such nestlings may face a higher risk of mortality immediately after fledging due to their weaker flying abilities (soft bones, shorter wings – Barclay 1994, McCarty 2001). Hence, proper knowledge of the actual developmental stage of a nestling prior to its leaving the nest would provide valuable information on its future performance.

As it is usually difficult to discriminate among fledglings at different developmental stages solely on the basis of morphology, there is a need for additional indicators. A potentially suitable indicator of nestling developmental maturity is the enzyme serum alkaline phosphatase (ALP), because its level varies with age (e.g., Viñuela et al. 1991, Hoffmann et al. 1994). Serum ALP activity is the sum of ALP activities from different organs: bone, liver and intestine. The two most relevant isoforms of this enzyme in the blood are liver-ALP and bone-ALP that are products of the same gene, but differ due to post-translational glycosylation (Hoffmann et al. 1994, Romagnoli et al. 1998). Liver-ALP is synthesised by hepatocytes (Sherlock and Dooley 2002). It reflects the function of ATPase (Ohkubo et al. 1974) and participates actively in the down-regulation of secretory activity of the intrahepatic biliary epithelium (Alvaro et al. 2000). Increased activity of liver-ALP in serum is typically associated with abnormalities in liver functions (Sherlock and Dooley 2002), but its level can also change with diet (Watkins et al. 2000) and with hormonal treatment (Solter et al. 1994). The bone isoform (bone-ALP) is a membrane-bound protein that is synthesised by ostebals in the bone tissue (de Behr et al. 2003), and may be a sensitive indicator of skeletal development in both mammals and birds (Price 1993, Hoffmann and Solter 1999, Allen et al. 2000, de Behr et al. 2003, Tilgar et al. 2004a, Smits et al. 2007).

In wild birds, ALP activity has been associated with bone growth in various bird species. Studies with raptors (Viñuela et al. 1991, Viñuela and Ferrer 1997, Bailey et al. 1998, Dobado-Berrios and Tella 1998, Villegas et al. 2002), the Pigeon Guillemot *Cepphus columba* (Seiser et al. 2000) and the White Stork *Ciconia ciconia* (Smits et al. 2007) show that blood levels of ALP tend to be maximum near the end of nestling period, when the growth of long bones is practically finished. Note that the above-mentioned studies, except Smits et al.

(2007), were based on total-ALP and provided no information about the bone-ALP isoenzyme. In passerine birds, ALP activity has been associated with nestling development only in few studies (total-ALP was measured by Ormerod et al. 1991, bone-ALP by Tilgar et al. 2004a, b). In contrast to large birds, there is some evidence that the rate of bone ossification (Bilby and Widdowson 1971, Ricklefs 1975) as well as bone-ALP level (Tilgar et al. 2004a) of small passerines decline gradually before fledging time. However, we still lack any reliable information about the age-related dynamics of bone-ALP in passerine birds.

The main objectives of this study were (i) to investigate age-related changes in the activities of ALP isoenzymes in the blood of growing nestlings, and (ii) to assess the suitability of bone-ALP as a marker of general nestling development. We assumed that bone-ALP activity predicts the rate of bone formation that, in turn, should be reflective of overall nestling maturity. Although liver-ALP is not indicative of bone mineralization, its level was also measured as potentially reflective of some other aspects of age-specific metabolism (e.g., Hoffmann et al. 1994, Alvaro et al. 2000 and references therein, Watkins et al. 2000). For these purposes, the nestlings of a small cavity-nesting passerine bird, the Great Tit (*Parus major*) were sampled at two distinct age points, in the middle (day 8) and near the end (day 15) of the nestling period. Specifically, we hypothesised that during the peak growth period (in the middle of the nestling period – Tilgar and Mänd 2006), bone-ALP level should be significantly higher than at the pre-fledging stage. Pre-fledging activity of bone-ALP was expected to correlate with the duration of the nestling period and wing length that is the nestling trait known to increase steadily near and beyond fledging time (e.g., Kunz and Ekman 2000). On the other hand, we expected pre-fledging tarsus length and body mass to be weakly related with bone-ALP activity because these traits are fully developed well before nestlings leave the nest (Ricklefs 1975, Kunz and Ekman 2000, McCarty 2001).

METHODS

The study was conducted near Kilingi-Nõmme (58° 7′ N, 25°, 5′ E), southwest Estonia, in 2004. The study area, containing both deciduous and coniferous forest plots, was *c*. 50 km^2. Great Tits bred in nest-boxes mounted on tree trunks at a height of 1.5–2 m. The internal size of nest-boxes was 11×11×30 cm and the diameter of the entrance hole was 3.5–4 cm. Old nest material was removed in March before the start of nest building. The nestboxes were checked daily by the end of incubation period (11–14 d) until all nestlings were hatched. In each brood, one synchronously hatched nestling was chosen randomly for measurements. On day 8 post-hatch it was weighed using a Pesola spring balance to the nearest 0.1 g, tarsus length was measured with a sliding calliper

to the nearest 0.1 mm, and a blood sample (c. 100 µl) was collected into heparinized capillary tubes from the brachial vein. These procedures were performed again on day 15 post-hatch (before the usual fledging time, which occurs between 17 and 19 days). In addition, wing length of the 15-day-old nestlings was measured with a sliding calliper to the nearest 1 mm. All samples were collected between 1000 and 1600 hours to minimize any possible variation in blood chemistries caused by the birds' daily rhythm (Ferrer [1990] and references therein). All broods were checked daily to determine the duration of the nestling period. Only successful nests (where at least one nestling fledged) were included in analyses.

Plasma was separated from blood cells by 10-min centrifugation at 10,000 rpm and stored at –20°C until analyzed. Total ALP activity was assessed as U/L by regular colorimetric test. The activities of bone- and liver-ALP were estimated with a standard agarose gel electrophoresis with an REP system (commercial REP Alkaline Phosphatase Isoenzyme Kit, Helena Laboratories Corp., Beaumont, U.S.A.). This procedure is based on different physicochemical and electrophoretic properties of isoenzymes. After electrophoresis, gels were incubated with REP ALP Isoenzyme Chromagen and then densitometrically scanned at wavelength of 595 nm. The control (Gel Alkaline Phosphatase Isoenzyme Control, Helena Laboratories) was used to aid in the identification of alkaline phosphatase isoenzymes by agarose electrophoresis. It was prepared from pooled human serum and contained a liver and bone isoenzyme band. The control was a stabilized liquid. The repeatability of ALP measurements was very high (bone-ALP: r=0.88, $F_{2,5}$=16.1, P=0.005; liver-ALP: r=0.95, $F_{2,5}$=38.4, P<0.001, total-ALP: r=0.96, $F_{2,6}$=54.0, P<0.0001, calculated as intra-class correlation coefficients from one-way ANOVA according to Lessells and Boag (1987).

Statistical package STATISTICA, version 7.1 (Statsoft, Tulsa, Oklahoma), was used for data analyses. Pairwise t-tests and Pearson correlations (referred to as 'r') were computed for normally distributed data, otherwise Spearman correlations (referred to as 'R_S') were used. Because no habitat-related differences in nestling body mass and tarsus length (*P*>0.16) and ALP-values (*P*>0.5) were found, combined data over habitats were used. All significance levels are for two-tailed tests.

RESULTS

Age-related dynamics of ALP.–Bone-ALP activity declined significantly from the 8th to the 15th day post-hatch, while only a weak tendency to decline was revealed in liver-ALP (Table 1).

Neither bone-ALP nor liver-ALP activity measured at day 8 predicted its level at day 15 significantly (bone-ALP: r=0.42, *P*=0.09, n=17, liver-ALP:

r=0.24, P=0.3, n=17). Bone-ALP was positively correlated with liver isoform at day 8 (r=0.55, P=0.014, n=19), but this relationship was not significant at day 15 (r=0.31, P=0.3, n=17).

ALP, nestling morphology and duration of nestling period.–Bone-ALP measured at day 15 was strongly positively correlated with the duration of the nestling period (Fig. 1, R_S=0.63, P=0.006, n=16), while this was not the case for liver-ALP (R_S=0.02, P=0.9, n=16). Neither of these isoenzymes measured at day 8 predicted the duration of the nestling period (bone-ALP: R_S=–0.20, P=0.4, n=17; liver-ALP: R_S=–0.24, P=0.35, n=17). All three morphological traits of the 15-day-old nestlings were (or nearly were) significantly and negatively correlated with the length of the nestling period (wing length, R_S= –0.49, P=0.047; tarsus length, R_S= –0.47, P=0.058; body mass, R_S= –0.48, P=0.051; n=16 for each trait). None of morphological traits measured at day 8 predicted the duration of the nestling period (tarsus length: R_S=–0.32, P=0.2, n=17; body mass: R_S=–0.38, P=0.13, n=17; wing length was not measured at this age).

Neither bone-ALP nor liver-ALP level correlated significantly with morphological traits of nestlings at day 8 (Table 2). At day 15, bone-ALP activity was inversely related to wing length and body mass, but not to tarsus length (Table 2). None of these body traits correlated significantly with liver-ALP at that age (Table 2).

DISCUSSION

Bone formation and ALP dynamics.–Bone-ALP activity measured in nestlings in the middle of the nestling period was higher than that measured a week later, shortly before fledging. Given that bone-ALP is produced by bone-forming osteoblast cells, the changes in its activity directly reflect bone formation (Cubo et al. 2000, Hamade et al. 2003). Therefore, declining values of bone-ALP observed in full-grown nestlings probably indicate the decelerating phase of skeletal ossification. This result is also consistent with our previous findings that bone-ALP level tends to decline prior to fledging, and the onset of this decline was postponed in the nestlings with reduced growth rates (Tilgar et al. 2004a, b). However, the dynamics of bone-ALP throughout the growth period can be different in birds with large body size. For example, in the White Stork, bone-ALP level tended to reach a maximum level shortly before fledging (Smits et al. 2007). Hence, intensive ossification may overlap with the active phase of somatic growth in small birds (e.g, in Great Tits the maximum mass gain occurs at day 6 – Tilgar and Mänd 2006), while these two processes appear to be temporally more separated in large birds, indicating that maximum mineralization in the latter species is predominantly related to appositional bone growth. However, further investigations are needed to clarify these observed differences among bird groups.

Another important finding of this study is that age-related changes in liver-ALP activity were less evident compared to respective changes in bone-ALP dynamics. This suggests that liver-ALP cannot be used as a sensitive indicator of maturity. It is still noteworthy that both bone and liver isoenzymes were positively correlated with each other in the middle of the nestling period, whereas this association was much weaker at the pre-fledging stage. Although liver-ALP activity is not related to skeletal development, this isoenzyme is susceptible to hormonal control (Solter et al. 1994) and dietary treatment (Watkins et al. 2000), thus potentially reflecting the overall intensity of metabolism. Hence, one can speculate that the intensity of metabolic processes is correlated with the rate of bone ossification at day 8, but not at the pre-fledging stage.

Bone-ALP as an indicator of nestling maturity.–Our previous studies indicated that ALP activity measured shortly before fledging can be used to reveal subtle developmental differences between the nestlings with similar sizes (Tilgar et al. 2004a, b). We proposed that young with faster growth rates may complete the rapid phase of ossification earlier than those with slower growth rates, and respective differences between developmental stages can be ascertained by different values of bone-ALP measured at the pre-fledging stage. We also suggested that measuring ALP in nestlings may help to predict the fledging date. Given that broods with faster growth rates usually fledge earlier than those with slower growth rates (Keller and van Noordwijk 1994, Viñuela and Ferrer 1997), we expected that the duration of the nestling period is closely linked to the pre-fledging maturity of nestlings. Indeed, this study demonstrated that bone-ALP level measured at day 15 was positively correlated with the duration of the nestling period. Moreover, we found that the pre-fledging activity of bone-ALP was inversely related to all three morphological measurements. This was expected in the case of wing length, because feather growth steadily increases up to, and even after, fledging time in tits (e.g., Kunz and Ekman 2000, Oddie 2000). On the other hand, body mass and tarsus length of nestlings usually stabilize several days before fledging in most passerine birds (Ricklefs 1975, Kunz and Ekman 2000, Oddie 2000, McCarty 2001, Tilgar and Mänd 2006). We emphasise that pre-fledging measurements were taken several days before the usual fledging time for Great Tits, when growth and development probably were not complete. However, although fledging date was also correlated with the morphological traits we measured in 15-day-old nestlings, these correlations were only marginally significant and considerably weaker than the correlation between fledging date and bone-ALP.

In conclusion, we suggest that the bone alkaline phosphatase level is a more reliable indicator of nestling maturity at the pre-fledging stage than body mass or structural measurements. Our most noteworthy finding is that bone-ALP correlates significantly with the fledging date of young Great Tits. Given that a prolonged nestling period is usually related to delayed development and poor

pre-fledging quality of nestlings, it is possible that measuring the level of this enzyme in the blood of nestlings just prior to fledging gives reliable information about their post-fledging survival prospects or future fitness.

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Table 1. Activities (U/L) of different isoforms of plasma alkaline phosphatase (ALP) in the blood of Great Tit nestlings measured at days 8 and 15 post-hatch (*n*=17 pairs).

	Day 8		Day 15		Paired *t*-test	
	Mean	SD	Mean	SD	*t*	*P*
Bone-ALP	170.5	64.0	129.3	43.1	2.82	0.012
Liver-ALP	858.9	222.9	751.9	108.8	1.82	0.09

Table 2. Correlations among three traits in Great Tit nestlings measured at days 8 and 15 post-hatch (*n*=17 at day 8 and *n*=16 at day 15).

	Wing length		Tarsus length		Body mass	
	r	*P*	*r*	*P*	*r*	*P*
Day 8						
Bone-ALP			0.30	0.24	0.34	0.18
Liver-ALP			0.08	0.8	0.08	0.8
Tarsus length					0.88	<0.001
Day 15						
Bone-ALP	−0.51	0.043	−0.43	0.095	−0.51	0.044
Liver-ALP	−0.44	0.085	−0.12	0.6	−0.06	0.8
Wing length			0.63	0.007	0.85	<0.001
Tarsus length					0.81	<0.001
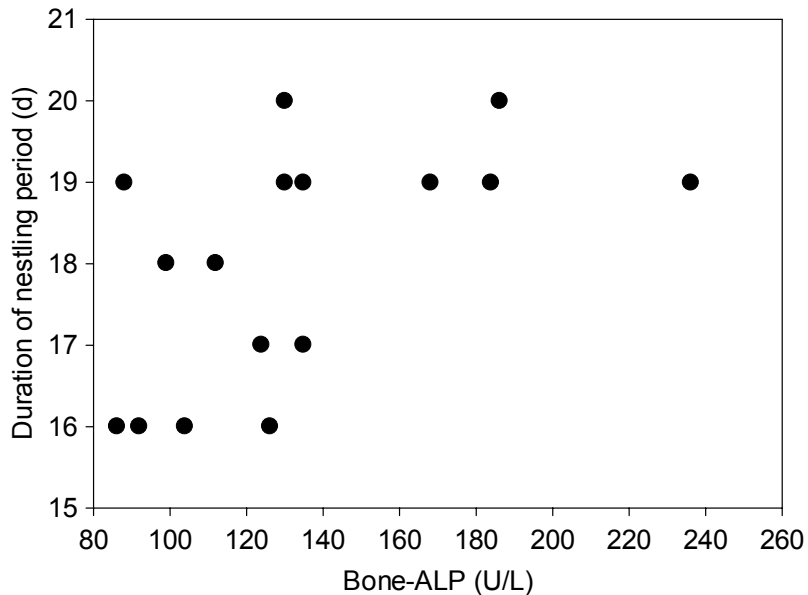
Fig. 1. The relationship between bone-ALP activity of nestlings at the pre-fledging stage (day 15 post-hatch) and the duration of the nestling period.

VII

Tilgar, V., Mänd, R., Ots, I., Mägi, M., Kilgas, P. & Reynolds, S. J. 2004.
Calcium availability affects bone growth in nestlings of free-living great tits
(*Parus major*), as detected by plasma alkaline phosphatase.
Journal of Zoology 263: 269–274.

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Kilgas, P., Mänd, R., Tilgar, V., Mägi, M., Leivits, A. Physiological costs of egg production and habitat variation in the hematological health state indices in great tits. 12–19. august 2006. 24th International Ornithological Congress. Hamburg, Germany. (poster).

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Kilgas, P., Tilgar, V., Mägi, M., Mänd, R. Blood parameters in Great Tits breeding in heterogeneous habitat: does preferred habitat make them sick? 27.07–1.08. 2002. 9th European Ecological Congress. Lund, Sweden. (poster).

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