DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
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HEALTH STATE INDICES OF REPRODUCING GREAT TITS (*PARUS MAJOR*): SOURCES OF VARIATION AND CONNECTIONS WITH LIFE-HISTORY TRAITS

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TARTU UNIVERSITY
PRESS

Institute of Zoology and Hydrobiology, Faculty of Biology and Geography, University of Tartu, Estonia

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Opponent: Dr. Benjamin Conrad Sheldon, University of Uppsala, Sweden

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers referred to their Roman numerals

I Hõrak, P., Jenni-Eiermann, S., Ots, I. & Tegelmann, L. 1999. Health and reproduction: sex-specific clinical profile of Great Tits (*Parus major*) in relation to breeding. — *Canadian Journal of Zoology* (in press).

II Ots, I., Murumägi, A. & Hõrak, P. 1998. Haematological health state indices of reproducing Great Tits: methodology and sources of natural variation. — *Functional Ecology* 12: 700–707.

III Ots, I. & Hõrak, P. 1998. Health impact of blood parasites in breeding Great Tits. — *Oecologia* 116: 441–448.

IV Hõrak, P., Ots, I. & Murumägi, A. 1998. Haematological health state indices of reproducing Great Tits: a response to brood size manipulation. — *Functional Ecology* 12: 750–756.

V Ots, I. & Hõrak, P. 1996. Great Tits *Parus major* trade health for reproduction. — *Proceedings of the Royal Society of London B* 263: 1443–1447.

VI Hõrak, P. & Ots, I. 1998. Health state and local survival in the Great Tit. — *Biol. Cons. Fauna* (in press).

INTRODUCTION

Evolutionary approach in contemporary animal ecology is based on the life-history theory, which uses the concept of resource allocation for explaining the coevolution of fitness-related traits. The theory relies on the concept of trade-offs which represent the costs paid in the currency of fitness when a beneficial change in one trait is linked to a detrimental change in another (Stearns 1992). Trade-offs emerge because organisms posses a limited amount of resources (energy, time) that they have to allocate between various activities in order to maximise the number of offspring produced during the lifetime (≈fitness).

The number and quality of offspring produced in every breeding attempt depends on the amount of resources that parents invest in reproduction (reproductive effort — Fig. 1). According to life-history theory, increasing current reproductive effort may diminish individual's probability of survival until the following breeding occasion (reproductive cost), because the resources invested in reproduction cannot be used for self-maintenance. Individuals behave optimally when they manage to divide their resources between various demands (e.g. reproduction and maintenance — Fig. 1 arrows 1 and 2) so that their lifetime reproductive output is maximised. Life-history theory predicts that individuals with a high probability of survival until the following reproductive occasion should not make too exhausting reproductive effort. However, when their probability of breeding again in the future is low, the optimal reproductive decision may be allocation of all resources, including those which could otherwise be spent on maintenance, for current reproduction, making a terminal reproductive effort.

Reproductive effort depends on individual's health state or condition, as individuals in a poor condition have less resources available for reproduction than individuals in good condition (state-dependent reproductive decisions — Fig. 1 arrow 3; McNamara & Houston 1996). On the other hand, reproductive effort might also affect condition (Fig. 1 arrow 4), as the amount of resources left for self-maintenance depends on reproductive effort. Condition is in turn associated with survival probability (Fig.1 arrow 5) which affects directly individual's fitness (Fig. 1 arrow 6). Yet another factor associated (through condition) with survival and reproductive effort is individual's susceptibility to parasite infections. Parasitized individuals might be in a poorer condition (Fig. 1 arrow 7) because fighting against parasite infections requires resources which could be used for other activities. A poor condition in turn makes individuals more susceptible for parasite infections allowing them to invade or relapse in the organism (Fig. 1 arrow 8).

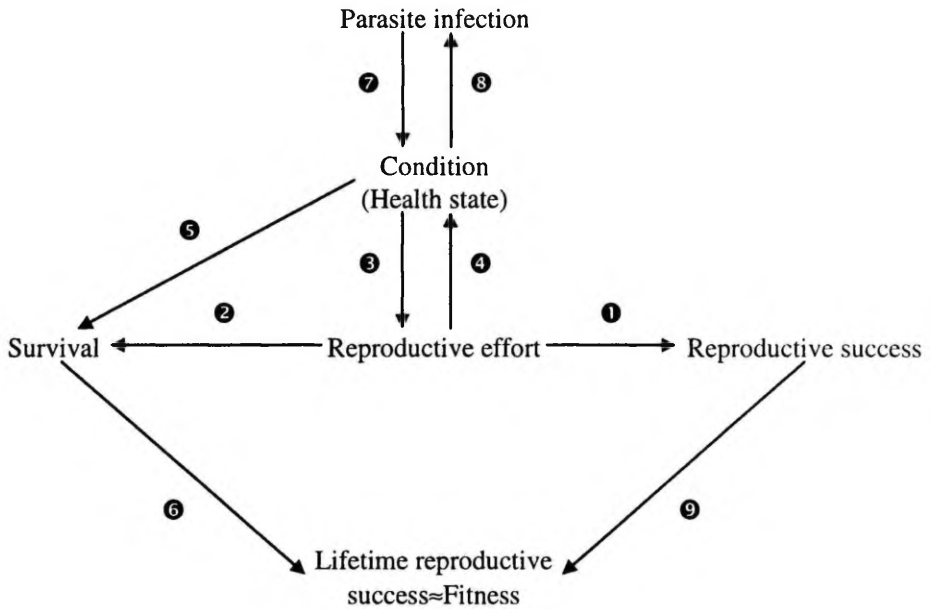

Figure 1. Possible relationships between parental condition (health state) and fitness. Arrows indicate dependent variables. The resources spent on reproduction during a current breeding event (reproductive effort) are linked to fitness through survival and reproductive success (1, 2, 6, 9). Individual's condition affects its survival probability (5) and reproductive effort (3), while intensive reproductive effort may in turn have a deleterious effect upon health state (4), enhancing organism's susceptibility to parasite infections (8). Parasites may affect fitness as for as infections may have a negative effect on the health state of the host (7).

The aim of this thesis is to study the connections between the components of fitness and individual's condition or health state on the example of the Great Tit (*Parus major*), by using simple clinical tests to measure various immunological and general health parameters. Different health state indices measure different aspects of individual condition and may also reflect health disorders of different duration. Moreover, there are several sources of variation not directly associated with the life-history components under study, such as seasonal and diurnal variations and differences due to sex and habitat, awareness of which is of crucial importance for distinguishing between environmental noise and the effect of experiment. To select appropriate research methods, consideration of all these aspects is of substantial importance. Therefore, my first purpose was to explain the sources of individual variation among different health state indices in order to test their suitability for application in field studies of passerine birds. Consequently, the first part of my thesis describes individual variation of simple immunological and general health parameters with

respect to breeding stage, sex, breeding area and time (diurnal variation). For this purpose, I described twelve (mostly hemato-serological) health parameters in Great Tits reproducing in two different (rural and urban) areas. These parameters include plasma protein concentrations, estimates of total and differential leukocyte counts, blood parasitemia, hematocrit and parental body mass.

The second part of my thesis addresses the question about relations between individual condition and reproductive effort and survival, concentrating on connections 2, 3, 4. 5, 7 and 8 as depicted in Fig. 1.

1. AN OVERVIEW OF HEALTH STATE INDICES

To estimate individual condition, I used twelve health state indices. These indices are divided into the following five categories: plasma proteins, total and differential leukocyte counts, blood parasitemia, hematocrit and residual body mass.

1.1. Plasma proteins

Blood plasma consists of many proteins the pattern of which reflects individual's physiological state. Decrease in total plasma protein concentration characterises almost all diseases, especially malnutrition. Physical exercise or water shortage, in turn, results in rise of the level of total plasma protein due to hemoconcentration (Kawai 1973). Of specific plasma protein levels (fractionated by using electrophoresis) I concentrated on albumin, gamma-globulin and total globulin.

Albumin is the biggest protein fraction in normal plasma and its main functions are to carry metabolites and other proteins, to act as an amino acid pool in protein synthesis and to serve as an energy resource when glycogen and lipid reserves are depleted. Decrease in albumin level is a symptom of almost every pathological processes while it reflects especially malnutrition, liver disorders and inflammation (e.g. Griminger & Scanes 1986).

The gamma-globulin fraction consists mainly of immunoglobulins (Ig) and therefore the concentration of gamma-globulins can be used as an estimate of humoral immune response to different antigens (e.g. Kawai 1973, White 1992). In case of stress, corticosteroids (stress hormones) can reduce gamma-globulin level by suppressing the activity of lymphocytes producing immunoglobulins (e.g. Leonard 1992).

In general, the globulin fraction of plasma consists mainly of acute phase proteins. The level of acute phase proteins increases immediately after injury or pathogenic challenge, which helps the organism survive during the period before specific immune response. Increase in total globulin level indicates acute inflammatory response (Kawai 1973).

In both acute disease and chronic infectious or inflammatory disease, diseased individuals reveal higher total globulin concentration and lower albumin/globulin (Alb/Glo) ratios as compared to healthy individuals (Kawai 1973, Griminger & Scanes 1986).

1.2. Total and differential leukocyte counts

Leukocytes protect the organism against various antigens. Increase in leukocyte number (leukocytosis) indicates inflammatory process and sometimes presence of a stressor. Usually, leukocytosis is caused by an elevated concentration of heterophils and lymphocytes.

Heterophils are non-specific phagocyting immune cells which migrate to tissues during inflammatory process. Due to release of free radicals and catabolic enzymes, heterophils can be harmful both to pathogens and to host's own tissues (e.g. Ling 1992).

Lymphocytes are highly specific immune cells that can be classified into two main types: T-lymphocytes and B-lymphocytes. T-lymphocytes originate from the thymus and their main functions are regulation of immune response and destruction of (especially intra-cellular) antigens. T-lymphocytes are considered to be the main part of cell-mediated immunity, while B-lymphocytes (originating from the bursa of Fabricius) contribute to humoral immunity. B-lymphocytes synthesise and secrete immunoglobulins, which are of crucial importance in antigen recognition (especially in case of exogenous antigens). In peripheral blood up to 80% of lymphocytes are T-lymphocytes and only ca 10% are B-lymphocytes. Hence, lymphocyte concentration obtained from peripheral blood estimates indirectly cell-mediated immunity (e.g. Parslow 1994). Decreased lymphocyte concentration in peripheral blood may signal immunosuppression which is assumed to make the organism more vulnerable to viral infections (e.g. Gross *et al.* 1980).

Stress index, widely used in case of poultry, is heterophile/lymphocyte ratio (H/L) which is known to increase in the presence of various stressors like infectious diseases, starvation and psychological disruption (Gross & Siegel 1983, Maxwell 1993).

1.3. Blood parasitemia

Blood parasites are mainly vector-transmitted protozoans living in the tissues and peripheral blood of their hosts and consuming a variety of host metabolites and haemoglobin (Garnham 1966). Blood parasite infection might be an important indicator of individual health state because parasite infection is expected to associate with individual's immunocompetence.

1.4. Hematocrit

Hematocrit or packed cell volume (PCV) measures the relative amount of red blood cells in total blood volume. Low values (anemia) are indicative of bacterial infections, gastrointestinal disorders, including parasitism and haemorrhage, or may reflect nutritional deficiencies of some minerals (Fe, Cu, Co). PCV values rise with increased oxygen consumption in case of high work load and with an elevated level of corticosteroids during acute stress (Sturkie & Griminger 1986).

1.5. Residual body mass

Body mass is affected by the multitude of components of individual state, which complicates the interpretation of patterns in its variation. However, it still deserves attention as a state index due to the easiness of its measuring and wide use in almost any study dealing with captured animals.

2. MATERIAL AND METHODS

2.1. Study species and area

The Great Tit is a small (ca 19 g), mainly insectivorous, short-lived and monogamous passerine bird. Only a small proportion of the fledged young reach breeding age, and more than half of those breed only once. The Great Tit is common on the whole Eurasian continent and accepts readily nestboxes for breeding, which makes it a very convenient subject for ecological research. As a result, there is a long tradition of research into Great Tit populations and much of their natural history is known.

The study was carried out in the town of Tartu (human population about 100,000) and in the rural area of Tõrvandi, located 5 km south of Tartu (58 22'N 26 43'E) during three years (1995–1997). The urban study area in Tartu consisted of four parks (about 22 ha) and avenues with a total length of 9 km. Nestboxes were mainly placed at roadsides with a distance of 30–40 m between them. The main tree species were *Tilia cordata, Acer platanoides, Betula pendula, Quercus robur*, and *Populus suaveolens*. Most streets in the urban study area bordered on gardens where winter feeding of birds was common.

The rural study area comprised two woods (Tõrvandi and Ropka, 2.5 km apart) surrounded by cultivated land. About two-thirds of the 72 ha area of Tõrvandi wood is covered with a moist birch forest, while the remaining third accounts for a poor mixed spruce forest; the tree species include *Picea abies, Pinus sylvestris, Betula pendula* and *Populus tremula*. The 550 ha Ropka wood is mostly covered with a rich mixed spruce forest with a deciduous understorey. In the understorey and at roadsides, *Corylus avellana* was the most common woody plant. In the rural study area nestboxes were placed at every 40–50 meters in lines (total length 11 km) running along forest edges and roadsides.

2.2. General methods

Breeding individuals were captured on their nests when their nestlings were 8 days old and a subset of individuals was recaptured on the fifteenth day of the nestling period for repeated measurement of health state indices (Paper II). In 1997, pre-breeding adult Great Tits were captured in town while roosting in nestboxes during three nights from 26 to 28 April (Paper I). Birds were sexed and classified as yearlings or older (≥2 years) according to plumage characteristics as described by Svensson (1992). They were weighed with a Pesola

spring balance with a precision of 0.1 g and their tarsi measured with a sliding caliper to the nearest 0.1 mm. To reduce the influence of structural size and diurnal variation, body mass was expressed as the residual from a multiple linear regression of mass on cubed tarsus length and weighting time (measured from sunrise). Only data for genuine first clutches were used.

For brood size manipulations, two two day old (day 0 = day of hatching) nestlings were moved from 'reduced' nests to 'enlarged' nests with a similar hatching date and clutch size, while the third nest with similar parameters was considered a control within the study site (Paper IV).

In paper V, brood size manipulation was conducted by removing two eggs from the clutch on the 7th day of incubation. Also, broods where more than one egg did not hatch were included in the category of 'reduced' broods.

2.3. Measurement of hemato-serological health state indices

Blood samples (up to 150 µl) for haematological measurements were taken from the tarsal or brachial vein. For identification of blood parasites and leukocytes, two individually marked thin blood slides were made by smearing a drop of blood at microscope slides. Thereafter, smears were air-dried, fixed in absolute methanol, and stained with azure-eosin. Slides were examined for the presence and abundance of blood parasites under 400× magnification for *Trypanosoma,* Microfilaria and *Leucocytozoon*, and under 1000× with oil immersion for *Haemoproteus* and *Plasmodium.* Scanning of a single slide for parasites took approximately 15 minutes. Because *Haemoproteus sp.* was the only common blood parasite, occurring in about 50% of the individuals, indices of parasitemia were confined to this genus.

The same slides were used for estimation of the total number and proportion of different types of leukocytes. The proportion of different types of leukocytes was assessed on the basis of an examination of a total of 100 leukocytes under oil immersion. Estimates of total white blood cell count (WBC) and intensity of parasitemia were obtained by counting the number of leukocytes or parasites per approximately 10,000 erythrocytes. Differential leukocyte counts were obtained by multiplying their proportions by WBC. In the analyses, only data for lymphocytes and heterophils as the most numerous immune cells were used. Scanning of a single slide for leukocyte proportions took approximately 15 minutes and for the WBC, 10 minutes.

Hematocrit was measured with digital callipers to the nearest 0.1 mm after 10 min centrifugation of heparinized capillary tubes at 10,000 rpm. The time lag between blood sampling and processing was usually 3–10 h, but never more

than 12 h. Meanwhile, the capillary tubes, sealed with wax at the bottom end and covered with rubber gap at the top, were left to stand in vertical position.

Plasma samples for protein electrophoresis were obtained by centrifuging blood in Microvette tubes (Sarstedt) for 10 minutes at 3000 rpm (Papers II, IV and VI) or by separating plasma from blood cells in the capillary tubes after determination of hematocrit (Papers I and III), after which the plasma was stored at –20°C until analysed. 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 scanned densitometrically at a wavelength of 525 nm. Total plasma protein concentration was determined by a photometric colorimetric test using the Biuret method. Because of difficulties in separating the prealbumin fraction from albumin, summed concentrations for both are reported and termed as albumin concentration.

3. RESULTS AND DISCUSSION

3.1. Sources of variation

3.1.1. Variation of condition due to stage of breeding (I)

Different stages of reproduction differ from each other in respect of environmental and physiological stressors imposed on individuals. During brood rearing, parents have high energetic requirement (e.g. Drent & Daan 1980), which might have a negative effect on the individual's condition due to increased work load. On the other hand, the nestling period is characterized by abundance of animal food which excludes nutritional limitations on parents. The pre-breeding period, in turn, can be more nutritionally limited, as the amount of insects is small. At the same time, there are no extra energetic costs related to brood rearing, which suggests diminutive health impact due to physical burden. Therefore, I studied how different clinical condition indices respond to these opposite environmental stressors (Paper I).

Compared to the brood-rearing period, Great Tits captured before egg-laying were on average 1.5 g heavier and had higher concentrations of total plasma protein and albumin (Fig. 2; Paper I). All these parameters indicate good nutritional condition, suggesting that the pre-breeding period is nutritionally not more limited compared to the nestling period, even though animal food is much less abundant in spring.

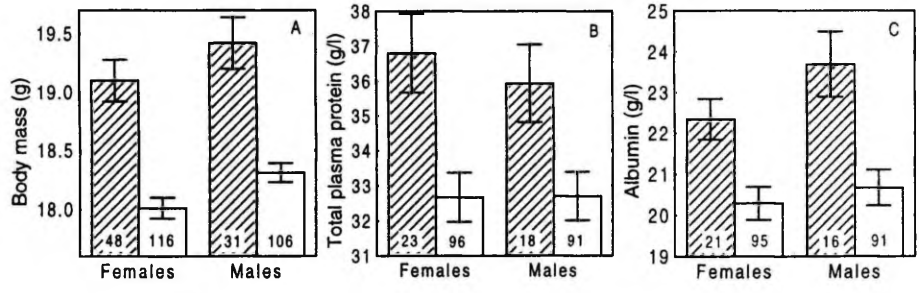

Figure 2. Nutrition indices of Great Tits in relation to sex and stage of reproduction. Data are presented as mean ±SE, hatched bars denote pre-breeding period and white bars the eighth day of nestling period. Numbers indicate sample sizes.

Gamma-globulin concentrations were higher in the pre-breeding period (Fig. 3). One possible explanation of this pattern could be that the infections activating humoral immune response are more severe in spring than during the nestling period in summer. Alternatively, humoral immune response could depend on individual's physiological condition. Barn Swallows (*Hirundo rustica*), whose

flight costs were increased by experimental tail elongation, did not reveal elevation of their gamma-globulin level after challenge with a novel antigen, while birds with shortened tails as well as controls responded through an elevated gamma-globulin level (Saino & Møller 1996). Therefore, one might speculate that elevated gamma-globulin levels during the pre-breeding period reflect trade-off between humoral immune response and reproductive effort as before egg-laying, birds seem to have more resources available for immune defence.

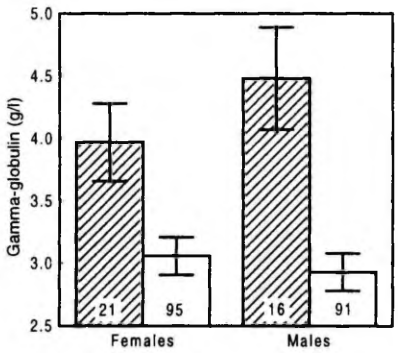

Figure 3. Gamma-globulins of Great Tits in relation to sex and stage of reproduction. Legend as in Fig. 2.

During the pre-breeding period Great Tits had higher hematocrit levels than in the breeding period (Fig. 4). This result could be explained by lower ambient temperature in spring, as high hematocrits in a cold period is expected to reflect increased oxygen-carrying capacity of blood for sustaining higher levels of thermogenesis (see e.g. Dawson & Bortolotti 1997).

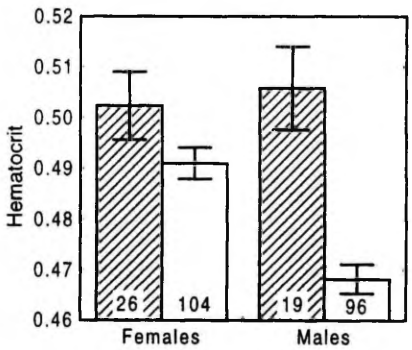

Figure 4. Hematocrits of Great Tits in relation to sex and stage of reproduction. Legend as in Fig. 2.

Only 7% of birds revealed *Haemoproteus* infection in peripheral blood before egg-laying. During the nestling period prevalence of blood parasite infection

increased to 45%. Relapse of *Haemoproteus* infection can be triggered by stress (e.g. Atkinson & van Riper 1991) or sex hormones (e.g. Haberkorn 1968), which probably accounts for seasonal increase in hemoparasite prevalence in the current study.

Heterophile/lymphocyte ratios were generally higher during the nestling period (Fig. 5 D), which suggests higher stress levels in Great Tits, accompanying increased work load during brood rearing (Paper I; see also Chapter 3.2.1.).

3.1.2. Individual persistence of condition indices during brood rearing

Avian ecologists often measure certain condition indices during some stage of the breeding cycle and interpret them in order to point to some persistent aspect of individual quality. In paper II, I addressed the question of temporal persistence of health state indices during the breeding period. Examination of correlations between trait values measured on the 8th and 15th day of the nestling period revealed that residual body mass and intensity of *Haemoproteus* infection were the most stable condition indices during the second half of the nestling period (Pearson correlations r=0.71 for both). Lymphocyte count, plasma albumin concentration, WBC, H/L ratio and total plasma protein concentration on the fifteenth day of the nestling period were moderately but significantly correlated with the values obtained on the eighth day of the nestling period (r=0.49, 0.42, 0.41, 0.33 and 0.30, respectively). These data suggest that no long-time extrapolations concerning individuals condition should be made on the basis of hematocrit, heterophile count or albumin/globulin ratio, as for as correlation coefficients between trait values, measured on day 8 and day 15 did not differ significantly from zero.

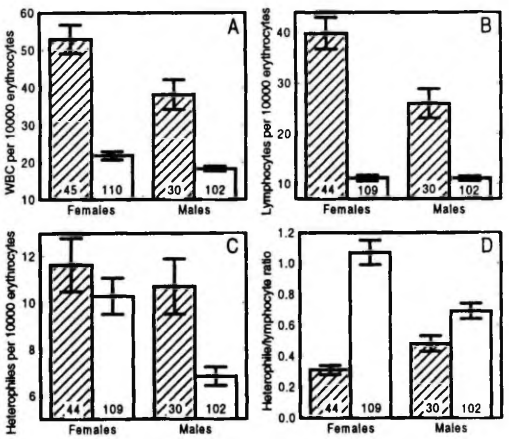

Figure 5. Absolute and differential leukocyte counts of Great Tits in relation to sex and stage of reproduction. Legend as in Fig. 2.

3.1.3. Sex differences of health state indices (I, II and III)

Besides differences caused directly by environmental changes, pre-breeding and brood-rearing individuals differ in respect of hormonal status, which might lead to sex-specific differences of health parameters. Testosterone levels in males are the highest in the period of mate and territory acquisition/guarding (e.g. Silverin 1990, Ketterson *et al.* 1991). Since testosterone is a well known immunosuppressant, its high levels might cause health deterioration (e.g. Bohus & Koolhaas 1991, Folstad & Karter 1992). Also, it is possible that in different periods one sex might be more stressed than the other due to a different share of breeding activities, which ought to lead to sexual differences between health state indices.

Indices reflecting individual nutritional condition (total plasma protein, albumin) did not differ between sexes in 1997 (Fig. 2 B and C; Paper I). However, in 1996, there was a tendency (albeit non-significant) of higher albumin levels in males in the middle of nestling period (Paper II). Absolute body mass of male Great Tits was larger than that of females (Table 1 in Paper I), but as the residual body mass of males and females did not differ, this was due to sexual dimorphism rather than to the better nutritional condition of males (Table 3 in Paper II).

Females had generally a lower albumin/globulin (Alb/Glo) ratio than males in 1997, while the same tendency was not statistically significant in 1996. A low Alb/Glo ratio is a typical symptom of pathological process, suggesting that females were more diseased than males.

In both the pre-breeding and the brood-rearing period, females had higher total leukocyte counts than males (Fig. 5 A). Before egg-laying, this difference was mainly the result of a higher lymphocyte count of females (Fig. 5 B). However, in the middle of the nestling period females had more heterophils than males (Fig. 5 C), which lead also to higher H/L ratios for breeding females compared to males (Fig. 5 D; Paper I). Similar sexual differences in leukocyte profiles were found also in 1996 (Paper II). In the pre-breeding period, on the contrary, H/L ratios for males were higher than for females (Fig. 5 D). The H/L ratio is considered an universal indicator of stress in poultry, and if this holds true also for passerine birds, then my results indicate that female Great Tits are more stressed than males in the middle of the nestling period, but males are more stressed during the pre-breeding period.

The result that males had less lymphocytes in the peripheral blood compared to females during the pre-breeding period might be caused by the effects of testosterone. Testosterone is a male gonadal hormone which suppresses immunity by affecting T-lymphocyte functions (e.g. Ahmed *et al.* 1985; Yamamoto *et al.* 1991; Wunderlich *et al.* 1992; Leitner *et al.* 1996). Therefore, it is supposed that males are more susceptible to parasite infections in spring than females. This hypothesis is consistent with the results showing that lymphocyte

count was affected by the infection status of *Haemoproteus* parasite only in males (Paper III). In the middle of the nestling period infected males had more lymphocytes in the peripheral blood than non-infected males, but such a pattern was not found in females (Fig. 6). Therefore, it is possible to speculate that males compensate for testosterone-induced immunosuppression in spring by stronger immune response against relapsed *Haemoproteus* infection in the middle of nestling period making a greater lymphopoietic effort compared to females.

No sex-related effects of humoral immune response (estimated as the level of gamma-globulins) against blood parasites were detected. This can be explained by different kinds of immune response against different stages of *Haemoproteus* infection: humoral immune response, which is considered less sensitive to testosterone, is directed against exo-erythrocytic stages (Graczyk *et al.* 1994, Wakelin 1997) and cell-mediated immunity controls relapsed infection in the bloodstream (Graczyk *et al.* 1994).

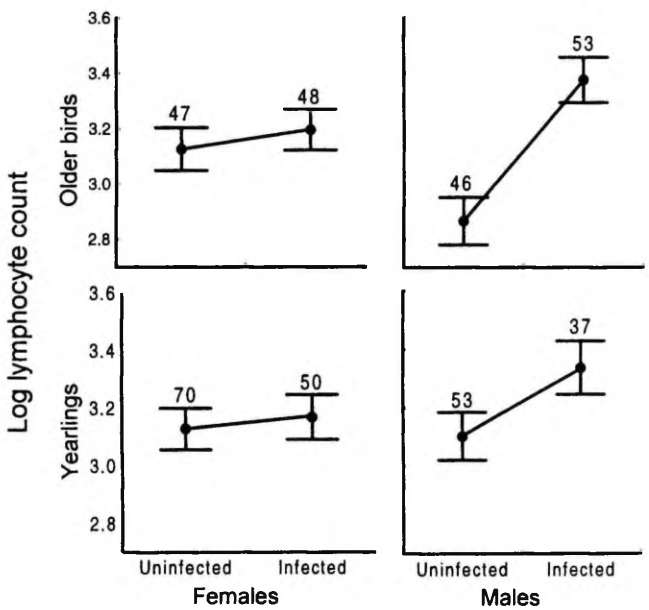

Figure 6. Lymphocyte hemoconcentration of Great Tits in relation to infection status, sex, and age. Data are presented as least square means and standard errors calculated by SAS GLM procedure, i.e., adjusted values, accounting for the confounding factors (from the ANOVA model: Lymphocyte count = *Haemoproteus*, Age, Sex, Time, Year, *Haemoproteus* × Age × Sex). Numbers indicate sample sizes.

No sexual differences were found in hematocrit values before egg-laying (Fig. 4), whereas during brood rearing, both in 1996 and 1997, females had higher hematocrits than males (Fig. 7.; Papers I and II). High hematocrits may

indicate elevated oxygen consumption accompanying high work load, which suggests that females may work harder than males during brood rearing (see discussion in Chapter 3.2.1.). Like several other studies (reviewed by Dawson & Bortolotti 1997) my results do not support the opinion that males have more erythrocytes than females because androgens stimulate and oestrogens suppress erythropoiesis (reviewed by Sturkie & Griminger 1986).

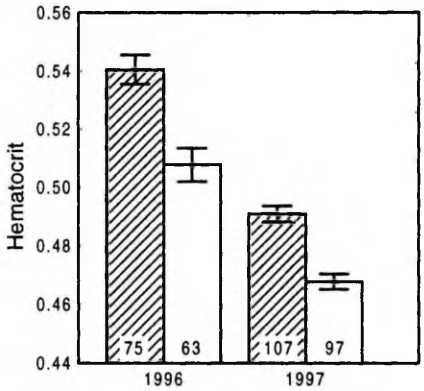

Figure 7. Hematocrit of Great Tits in relation to year and sex. Data are presented as mean ±SE, hatched bars denote females and white bars males. Numbers indicate sample sizes.

I found no sex difference in the prevalence of *Haemoproteus* infection in 1997 (Paper I), but in 1996, brood-rearing females had higher intensities of parasitemia (Paper II). However, the latter finding may not be typical because there was no sex difference in *Haemoproteus* intensities in same study area in 1995 (Ots 1996).

3.1.4. Differences of health state indices between study-sites (II and III)

On the basis of higher total protein and albumin level (Paper II, p. 704), urban males can be considered to be in better condition than rural males. Both female and male rural Great Tits revealed higher hematocrits than tits in the urban population (Paper II, p. 704). This phenomenon may have two possible explanations. First, urban birds are exposed to lead pollution from traffic exhaust fumes, and their lower hematocrits are caused by lead-induced suppression of hemopoiesis (see e.g. Kostelecka-Myrcha *et al.* 1997). Second, because rural Great Tits appear to make generally a greater reproductive effort (Hõrak 1995), their higher hematocrits can be associated with exercise-induced polycythemia due to high work load (see also Chapter 3.2.1.).

In two of the three study years, *Haemoproteus* infection was associated with a larger body mass in the urban but not in the rural study area (Fig. 8). The tendency for infected individuals to be heavier than uninfected individuals suggests that infected tits avoid losing their body mass in order to save resources for combating parasite infection. This result supports indirectly the hypothesis that birds may benefit from reduced flight costs accompanied with low body mass, and save resources for self maintenance and food provisioning (Freed 1981, Norberg 1981). If mass change represents an optimal balance between the costs and benefits of low body mass (Nur 1984, Hillström 1995), then uninfected healthy individuals can evidently afford to reduce their body mass to a greater extent than diseased individuals. The population differences of body mass changes can be explained by the different reproductive tactics of rural and urban birds: possibly, infected rural Great Tits, unlike urban tits, are less prone to invest their resources in parasite defence because they are more inclined to making terminal reproductive effort (see also Chapter 3.2.1.).

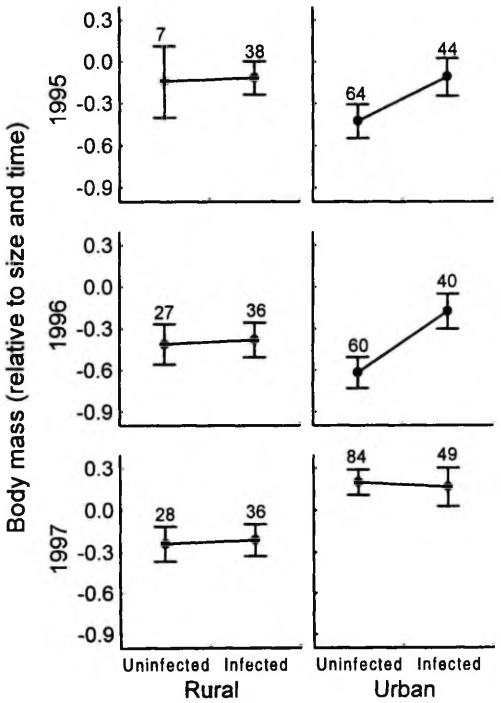

Figure 8. Residual body mass (Mean ± SE; adjusted for body size and weighing time) of Great Tits in relation to infection status, year and study site (in rural or urban habitat). Legend as in Fig. 6.

3.1.5. Diurnal variation of health state indices (II)

Significant diurnal variation appeared only in parameters associated with leukocyte counts (Table 1). Females roosting in nestboxes had higher numbers of lymphocytes and heterophils which also caused higher total leukocyte count compared to females sampled during day-time. A possible reason for higher counts of leukocytes in the night might be allocation of resources, used for other activities in day-time (Ricklefs 1979, Starck 1996), to cell proliferation.

Table 1. Health state indices of female Great Tits captured while roosting in the nestboxes ('Night') and feeding nestlings ('Day'). Leukocyte and parasite counts are expressed as number/10000 erythrocytes.

Trait	Night Mean±SD (n)	Day Mean±SD (n)	p diff.
Total plasma protein (g/l)	33.6±5.0 (10)	33.5±9.7 (67)	0.525[a]
Albumin (g/l)	20.9±4.7 (7)	20.4±4.7 (46)	0.799[a]
Albumin/Globulin ratio	1.7±0.5 (7)	1.8±0.6 (46)	0.358[a]
WBC	96.6±24.4 (11)	50.7±23.6 (76)	<0.001[a]
Lymphocyte count	58.4±20.4 (11)	26.8±15.0 (76)	<0.001[a]
Heterophile count	37.8±17.6 (11)	23.7±14.5 (76)	0.011[a]
H/L ratio	0.72±0.42 (11)	1.12±1.02 (76)	0.174[a]
Haemoproteus intensity	19.7±15.6 (7)	101.0±376.0 (29)	0.924[a]
Hematocrit	0.56±0.04 (6)	0.54±0.05 (65)	0.216[b]
Residual body mass	−0.41±0.69 (11)	−0.51±0.84 (77)	0.703[b]

[a] — Mann-Whitney U-test, [b] — t-test

3.2. Connections with life-history traits

3.2.1. Effect of reproductive effort on health (IV and V)

If individuals pay the cost of reproduction in terms of deleterious effects on their health state, then intense reproductive effort must result in poor condition due to the effect of high work load on health. Indeed, brood size manipulation affected significantly lymphocyte count (Fig. 9) and the heterophile/lymphocyte ratio (Fig. 10; Papers IV and V). Decreased lymphocyte hemoconcentration in rural Great Tits tending increased broods suggest that reproductive effort might cause immunosuppression, which would enable microorganisms to escape immunological reaction and establish or relapse infection.

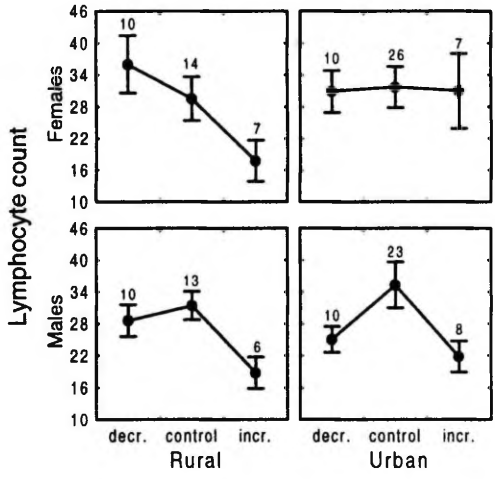

Figure 9. Effect of brood size manipulation on parental lymphocyte count in two Great Tit populations. Decr. stands for decreased broods and incr. for increased broods. Untransformed data, presented as mean±SE. Numbers indicate sample sizes.

The heterophile/lymphocyte ratio is a widely used stress index in poultry (Gross & Siegel 1983, Maxwell 1993), which is known to increase in the presence of almost any kind of stressors. The increased H/L ratio in response to brood enlargement (Fig. 10; Paper IV) and the highest H/L ratios for unmanipulated individuals making greatest reproductive effort, measured as total prefledging brood weight (Table 2; Paper V), indicate that intensive reproductive effort might be a cause of stress syndrome.

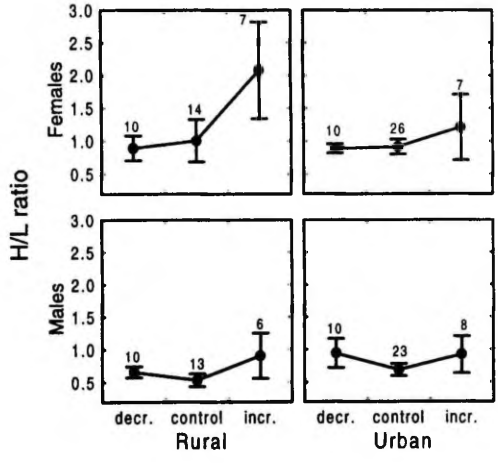

Figure 10. Effect of brood size manipulation on parental heterophile/lymphocyte ratio in two Great Tit populations. Legend as in Fig. 9.

Table 2. Spearman rank correlations (r_s) between H/L ratios and reproductive effort, measured as total prefledging brood mass in Great Tits in 1995.

	unmanipulated	all broods
females+males	r_{s23}=0.62, p<0.001	r_{s51}=0.41, p=0.002
females	r_{s12}=0.50, p=0.064	r_{s29}=0.34, p=0.064
males	r_{s9}=0.64, p=0.035	r_{s20}=0.42, p=0.051

In addition to leukocyte profiles, brood size manipulation affected also the hematocrit of breeding Great Tits. Hematocrit increased in response to brood enlargement (Fig. 11; Paper IV); the reason of this phenomenon might be exercise-induced polycythemia (e.g. Hsia *et al.* 1995, Toll *et al.* 1995, Wu *et al.* 1996, Piersma *et al.* 1996) which is caused by elevated oxygen requirement during increased work load. This explanation is supported by the fact that hematocrits were the highest among increased broods of rural Great Tits (Fig. 11) which, contrary to urban birds, fledged also more nestlings than controls, and were thus likely to work most intensively. This result is consistent with the correlation between elevated hematocrit and increased work load found in Barn Swallows (Saino *et al.* 1997).

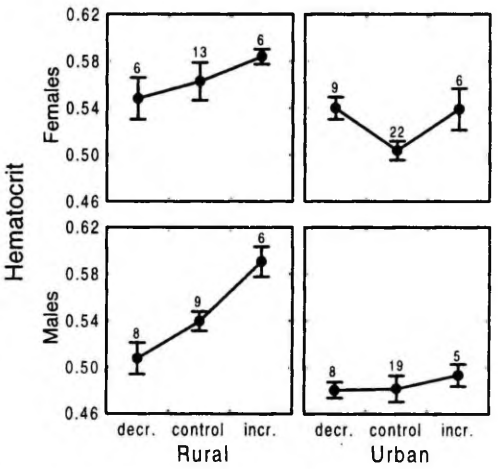

Figure 11. Effect of brood size manipulation on parental hematocrit in two Great Tit populations. Legend as in Fig. 9.

Besides haematological parameters, brood size manipulation affected also parental body mass (Fig. 12; Paper IV). Although the experimental effect was not significant in a two-tailed test ($F=2.6$, $DF_{2,139}$, $p=0.081$), the difference had an expected direction, suggesting that mass loss accompanied increased work load.

Plasma proteins and estimates of total leukocyte and heterophile count were not affected by brood manipulation. This suggests that elevated work load was not accompanied with serious pathological processes.

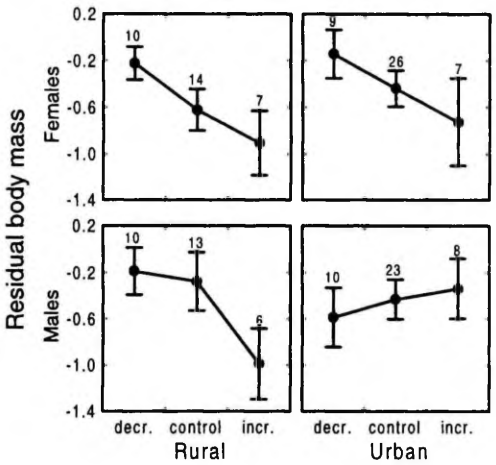

Figure 12. Effect of brood size manipulation on parental body mass (residual in respect to size) in two Great Tit populations. Legend as in Fig. 9.

The intensity of *Haemoproteus* infection was not affected by brood size manipulation in 1996 (Paper IV). This result differs from those of previous Great Tit studies which established the effect of reproductive effort on hemoparasite infections (Norris *et al.* 1994, Allander 1997, Richner *et al.* 1995). Moreover, in the previous (1995) year experimental reduction of brood size in the same populations resulted in decreased *Haemoproteus* parasitemia among rural female Great Tits (Paper V). One possible explanation for a such an inconsistency could be that the year 1996 was extremely favourable for breeding, and rearing of unmanipulated broods might have demanded less effort than in the previous year. Besides, the relationship between parasitemia and reproductive effort may be obscured because individuals appear unparasitised not only because of effective immune response but also because they are (occasionally) not infested by vectors (blood-sucking Diptera).

Most effects of brood size manipulation on health parameters in 1996 (Paper IV) were manifested only in the rural but not in the urban population (Fig. 9–12). Similar results were obtained also in 1995 when the relationship between reproductive effort and *Haemoproteus* parasitemia revealed only in rural Great Tits (Paper V). These results are interesting because brood size manipulation by two nestlings should have a proportionally stronger effect in town where clutch sizes were on average by two eggs smaller than in the rural population.

One possible explanation why brood size manipulation affected only rural Great Tits could be the generally poorer condition of urban birds, which obscures detection of such effects in town. Urban tits are probably more seriously food-limited during breeding because their clutches are by two eggs smaller, and they also fledge three nestlings less than their rural conspecifics (Hõrak

1993a). Occurrence of seasonal decline in egg size (Hõrak *et al.* 1995) and clutch size (Hõrak 1993b) in urban but not in rural Great Tits also suggests that urban birds are subjected to more severe proximate reproductive constraints. This explanation, however, is contradicted by the finding that urban tits have higher albumin level than rural tits (Chapter 3.1.4.; Paper II), which indicates a good nutritional state of urban birds. An alternative explanation for the different responsiveness of rural and urban Great Tits to brood manipulation would be that rural tits possess reproductive tactics with a greater propensity of investing in current reproductive attempt. Clutches of rural Great Tits are among the largest recorded for a species (11.1 eggs), which may indicate extremely intense reproductive effort in this particular population (Hõrak *et al.* 1995). Notably, inter-generation reproductive costs, indicated by the positive correlation between female survival and nestling mortality, appeared only among rural Great Tits but not in Tartu (Hõrak 1995), where adult survival was also generally higher (Hõrak & Lebreton 1998). It is thus possible that rural Great Tits are more prone to invest their maintenance resources in current reproductive attempt and make a terminal reproductive effort, since their probability to survive until the following breeding season is low anyway.

3.2.2. Health state and local survival (VI)

The concept of reproductive costs is based on the assumption that the effort devoted to current reproduction may reduce individual's chances to survive and reproduce successfully in the future. Hence, individuals with poor health, resulting from high reproductive effort, should have low probability of survival.

The results of this study did not reveal any relationship between non-parasitological health state indices and local survival. This suggests that parameters that appeared to be associated with reproductive effort, namely lymphocyte concentration in the peripheral blood, heterophile/lymphocyte ratio and hematocrit, reflected only transient changes in individual's physiology. Therefore, the state indices that were affected by brood size manipulation experiment probably do not reflect any persistent effect of reproductive effort upon individual condition.

The only condition index that was associated with local survival rates was prevalence of *Haemoproteus* blood parasite infection in yearling Great Tits. Of the uninfected yearlings 33% were recaptured as breeders in the following years, whereas only 12% of infected yearlings were recaptured (Fig. 13).

This result can have two possible explanations: 1) either birds succumbed due to *Haemoproteus* infection *per se*, or 2) generally poor health state of those individuals that were likely to die after reproduction was associated with *Haemoproteus* infection as a by-product. The first possibility seems more likely because it is consistent with the age-dependent effects of the prevalence of

Haemoproteus on local survival. Lack of the effect of infection on local survival in older age classes can be accounted for the higher virulence of the parasite in young, immunologically naive birds. This explanation is compatible with the finding that although in both age classes infected individuals had gamma-globuninemia, yearlings had generally lower gamma-globulin concentration than older birds (Fig. 14; Paper III). This suggests that older individuals probably have more efficient humoral immune response than yearlings.

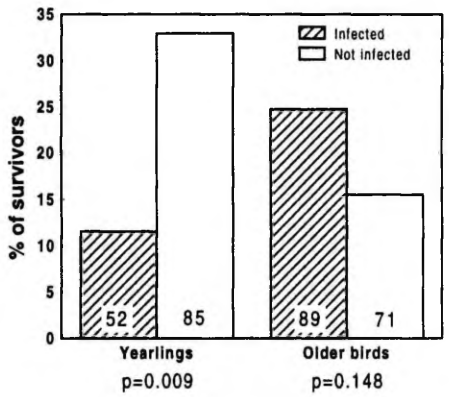

Figure 13. Relationship between local survival and prevalence of *Haemoproteus* in yearling and older Great Tits. Data pooled over 1995 and 1996; individuals that were sampled in both years are included only once. Difference in prevalence between survivors and non-survivors is significant in yearlings (χ^2_1=6.82, p=0.009) but not in older birds (χ^2_1=1.53, p=0.148).

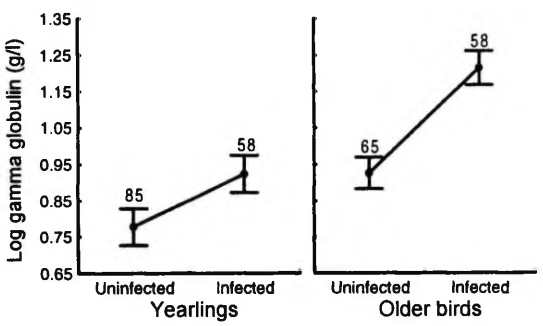

Figure 14. Plasma gamma-globulin concentration (Mean ± SE) of Great Tits in relation to infection status and age. Legend as in Fig. 6.

Cell-mediated lymphocytic immune response to *Haemoproteus* infection differed also in yearlings and in older male Great Tits (see Fig. 6; Paper III). Old uninfected males showed the lowest lymphocyte count, which could be ex-

plained by the generally stronger cell-mediated immune response (to other diseases) of yearlings. Young birds may have stronger cell-mediated immune response either because they are more susceptible to infections for immunogenetic reasons (selective mortality), or because older birds have acquired immunity during their longer exposure to pathogens. Initial hemoparasite infections often appear deadly, which makes young age classes most susceptible, but if the host survives, the parasite may enter a chronic phase with little or no signs of disease (Atkinson & van Riper 1991). It is therefore possible, that most of the adult population carries chronic infections and significant mortality occurs only in the young non-immune cohort. This explanation would also be compatible with observations that young birds usually have lower prevalence but higher intensity of blood parasites (Davidar & Morton 1993, Allander & Bennett 1994, Norris *et al.* 1994, Merilä *et al.* 1995, Merino & Potti 1995, Sundberg 1995, Ots 1996).

3.2.3. Health state indices and physiological mechanisms of reproductive costs

Do the health state indices examined in this study appear suitable candidates for explaining the physiological mechanisms of reproductive costs? To fulfil this criterion, a trait has to 1) depend on reproductive effort and 2) be associated with the host's future survival or fecundity.

The result that brood size manipulation affected markedly haematological traits only in one of the two Great Tit populations (Papers IV and V) indicates that connections between rearing broods of manipulated size and parental physiology are by no means ubiquitous. In certain environmental conditions (e.g. unfavourable food situation) individuals behave optimally when they do not increase reproductive effort in response to brood size enlargement (Tammaru & Hõrak 1999). This result implies the necessity of studying the physiological effects of reproductive effort in populations breeding under different environmental conditions. Even so, the responsiveness of individuals to brood size manipulation may differ within the same population in different years, as indicated by the presence of the effect of experiment on *Haemoproteus* parasitemia in the rural study area in 1995 (Paper V) but not in 1996 (Paper IV). Notably, there are also some inconsistencies among the results of other studies demonstrating the effect of brood size manipulations on haematozoan infection. Only one study (Allander 1997) established that brood size manipulation had similar effects on *Haemoproteus* infection in both male and female Great Tits. Richner *et al.* (1995) and Norris *et al.* (1994) showed that brood enlargements resulted in increased parasitization in male but not female Great Tits, while in this study (Paper V) the experimental effect was revealed only in females.

Thus, if brood size manipulation does not necessarily lead to increased reproductive effort in individuals rearing increased broods (Tammaru & Hõrak 1999), inconsistencies of brood size manipulation effects on Great Tits in this study could be attributed to differences in study areas and years. Another potential reason for such an inconsistency of effects in brood size manipulation on parental parasite loads might be that some birds did not respond to increased reproductive effort by increased parasitemia because they were not (occasionally) infected by vectors.

At present, it is difficult to say anything about the validity of the assumption that the studied health parameters are related to residual reproductive value of the host. The only condition index that was associated with local survival rates was prevalence of *Haemoproteus* blood parasite infection in yearling Great Tits (Paper VI). However, although the results of my study indicate the impact of *Haemoproteus* on the survival of yearlings (Paper VI) and the negative effect of *Haemoproteus* on host's health in all age classes (Paper III), the brood size manipulation experiments did not yield clear cut results concerning connections between reproductive effort and parasitism.

SUMMARY

Most of the health state indices studied on Great Tits showed significant variation between the pre-breeding and nestling period of reproduction which may be the result of different environmental and physiological stressors imposed on individuals in different stages of breeding. Only three health parameters proved inconstant between the 8th and 15th day of nestling period. In addition to effect of the environment, individuals may differ in respect of hormonal status, which was probably reflected in sex-specific differences in health parameters. In general, males appeared to be more stressed than females during the pre-breeding period, whereas an opposite situation occurred in the nestling period. The possible immuno-suppressive effect of testosterone is suggested by the result that *Haemoproteus* infection was associated with higher lymphocyte counts only in males but not in females. All health state indices associated with leukocyte counts (except the H/L ratio) revealed significant diurnal variation. Another source of variation in individual condition was linked with breeding sites located in different habitats. Tits breeding in an urban area were generally in better condition as compared to birds breeding in a rural area. One possible explanation for such a phenomenon might be the possibility that rural Great Tits invest less resources in self maintenance and are more prone to make terminal reproductive effort than their urban conspecifics.

Brood size manipulation affected the lymphocyte counts of parents, which suggests that high work load is associated with immunosuppression. According to elevated H/L ratios, the individuals rearing increased broods appeared to be more stressed than birds of other experimental categories. High work load was accompanied also with elevated hematocrit and reduced body mass. *Haemoproteus* infection was affected by brood size manipulation in 1995 but not in 1996.

Only one condition index was associated with local survival. Of the yearlings uninfected with *Haemoproteus* 33% were recaptured as breeders in the following years, but only 12% of the infected yearlings were recaptured. Lack of such survival differences in older birds, as well as age-related differences in gamma-globulin concentration and lymphocyte counts between infected and uninfected birds, suggest that immunologically naïve young birds are more susceptible to infections than birds of older age classes.

None of the health parameters under study appeared to be perfectly suitable for the explanation of physiological mechanisms of reproductive costs, as far as no condition index was consistently associated with both reproductive effort and local survival. *Haemoproteus* infection appeared to be the only health parameter which fulfilled, to a certain extent, the criteria necessary for explaining of the mechanisms of reproductive costs.

KOKKUVÕTE

Pesitsevate rasvatihaste (*Parus major*) tervisliku seisundi indikaatorid. Looduslik varieeruvus ja seosed elukäiguomadustega

Mikroevolutsiooniliste uuringute paradigmaatiliseks karkassiks on tänapäeval elukäiguteooria (*life-history theory*). Elukäiguteooria kohaselt on elukäiguomadused (*life-history traits*), nagu järglaste arv ning suurus, eluiga, suguküpsusiga, vanusest ja soost sõltuvad sigimisinvesteeringud ning suremus jm., koevolutsioneerunud mitmesuguste ökoloogiliste probleemide (nt. paljunemine, kaitse, stressitolerants) lahendamiseks. Elukäiguteooria rakendamisel on oluline selgitada, kuivõrd peegeldab isendite fenotüübiline varieeruvus geneetiliselt fikseeritud elukäigustrateegiate mitmekesisust ja kuivõrd keskkonnast tingitud piiranguid. Selline käsitlusviis põhineb isendite seisundierinevuse kontseptsioonil, mis käsitleb sigimisotsuseid ning nende seost kohasusega isendi tervisliku seisundi e. konditsiooni funktsioonina. Seisundierinevustel põhinevate realistlike elukäigu evolutsiooni mudelite testimise eelduseks on sobivate konditsioonikomponentide väljaselgitamine. Ideaalne konditsiooniindeks peab 1) olema välitingimustes mõõdetav, 2) peegeldama isendi seisundi võimalikult pikka aega püsivat ning integreeritud aspekti, 3) sõltuma sigimispingutusest (*reproductive effort*) ja 4) seonduma kohasusega. Eelnevast lähtudes oli dissertatsiooni eesmärgiks selgitada erinevate (peamiselt hemato-seroloogiliste) tervisliku seisundi indikaatorite e. konditsiooniindeksite varieeruvuse komponente ning võimalikke seoseid elukäiguomadustega (jn. 1) rasvatihase kui mudelliigi näitel.

Kõigil leukotsüütidega seotud tervisliku seisundi indikaatoritel (v.a. heterofiilide/lümfotsüütide suhe) ilmnes statistiliselt oluline ööpäevane varieeruvus. Linnas pesitsevad tihased olid üldiselt paremas tervislikus seisundis, võrreldes metsas pesitsevatega. See tulemus on kooskõlas hüpoteesiga, mille kohaselt metsa-populatsiooni tihased investeerivad sigimisse rohkem ressursse kui nende urbaniseerunud liigikaaslased.

Füsioloogilise stressi sümptomid olid pesitsuseelsel perioodil püütud isastel rasvatihastel tugevamad kui emastel, vastandina poegade toitmise faasile, kus emased olid suuremas stressis. See tulemus on tõenäoliselt selgitatav hormonaalse profiili ning pesitsusaegse tööjaotuse sugudevahelise erinevusega. Potentsiaalne testosteroonist tingitud immuunsupressioon võib selgitada, miks *Haemoproteus*-vereparasiitide nakkus oli seotud suurenenud lümfotsüütide kontsentratsiooniga ainult isastel, mitte emastel tihastel.

Rasvatihaste tervislik seisund erines oluliselt pesitsuseelsel ja poegade toitmise perioodil, mille põhjuseks võib olla keskkonnatingimuste ja füsioloogiliste

stressorite muutumine pesitsusperioodi jooksul. Uuritud konditsiooniindikaatorite individuaalne varieeruvus poegade toitmise perioodi 8. ja 15. päeva vahel oli enamiku (9/12) tunnuste puhul väiksem isenditevahelisest varieeruvusest.

Pesakonna suuruse manipuleerimine mõjutas vanemate lümfotsüütide kontsentratsiooni, mida võib tõlgendada kui suurest töökoormusest tingitud immuunsupressiooni. Suurendatud pesakondadega isenditel oli ka kõrgem heterofiilide/lümfotsüütide suhe, mis näitab nende lindude suuremat stressi, võrreldes kontroll- ja vähendatud pesakondi kasvatavate isenditega. Eksperimentaalselt suurendatud töökoormusega kaasnes ka kõrgem hematokrit ja madalam kehakaal. Pesakonna suuruse manipuleerimine mõjutas *Haemoproteus* sp. nakkust 1995. aastal, kuid mitte 1996. aastal.

Ainult üks uuritud konditsiooniindeksitest seostus tihaste lokaalse ellujäämusega. 33% vereparasiitidega nakatumata aastastest lindudest pesitses järgnevatel aastatel, samas kui nakatunud noorlindudel oli see näitaja ainult 12%. Sellise erinevuse puudumine vanematel isenditel ja vanusest tulenevad erinevused nakatunud ja nakatumata lindude gammaglobuliinide ja lümfotsüütide kontsentrantsioonis näitavad, et lõplikult kujunemata immuunsüsteemiga noorlinnud on nakkuste suhtes tundlikumad kui vanemad linnud.

Ükski uuritud terviseparameetritest ei osutunud universaalselt sobilikuks sigimishinna (*reproductive cost*) füsioloogiliste mehanismide seletamiseks, sest ükski tervisliku seisundi indikaator ei olnud seotud ühel ja samal ajal nii sigimispingutusega (*reproductive effort*) kui ka ellujäämisega. Kõige paremini vastas sigimishinna mehanismide seletamise kriteeriumitele *Haemoproteus* sp. nakkus.

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HEALTH AND REPRODUCTION: SEX-SPECIFIC CLINICAL PROFILE OF GREAT TITS (*PARUS MAJOR*) IN RELATION TO BREEDING

P. Hõrak*, S. Jenni-Eiermann**, I. Ots*** and L. Tegelmann***

**Estonian Institute of Zoology and Botany, Riia 181, Tartu 51014, Estonia*
***Swiss Ornithological Institute, CH-6204 Sempach, Switzerland*
****Institute of Zoology and Hydrobiology, Tartu University, Vanemuise 46, Tartu 51014, Estonia*

Abstract

To describe the changes in clinical profiles of individuals that occur during different stages of reproductive cycle, seventeen condition indices including leukocyte counts, plasma proteins and metabolites, hematocrit and hemoparasite prevalence, were examined in pre-laying and brood rearing great tits (*Parus major*). The metabolic profile pointed to an increased fat metabolism during the pre-breeding period. The protein metabolism did not indicate a nutritional limitation. Pre-laying birds had elevated gamma-globulin levels which may indicate either their higher investment to humoral immune defence, or alternatively, greater exposure to immune challenge as compared to breeders. Sexes did not differ in respect to prevalence of hemoparasites but females had generally lower albumin/globulin ratios than males which might indicate their greater disposition to infectious diseases. Breeding females had higher hematocrits and heterophile/lymphocyte ratios than males, suggesting that brood rearing exerts greater work load and stress to females. In contrast to the breeding period, males seemed to be more stressed than females prior to egg-laying, as indicated by their lower lymphocyte counts and higher heterophile/lymphocyte ratios.

Introduction

Evolutionary biologists since Darwin (1871) and Fisher (1930) know that individuals' properties of environmental origin, such as health and vigour may affect various components of fitness. The impact of inter-individual variation with respect to these properties, commonly referred as phenotypic quality, nutritional state, or condition, also plays a central role in recent developments of evolutionary animal ecology, where the concept of individual condition has become an indispensable component of state-dependent models of reproductive effort (e.g., van Noordwijk and de Jong 1986; Price *et al.* 1988; McNamara and Houston 1992, 1996). Nevertheless, despite a credit given to state- or condition-dependent approach in life-history models, the question, what actually constitutes condition and how should it be measured in the field, remains still poorly explored.

Recently, good progress in solving these problems has been made by application of simple clinical screening methods, conventional to human and veterinary medicine, for assessment of the health state of wild animals. Such an approach enables one to describe

various nutritional and immunological characteristics of an individual on the basis of information obtained from the minute blood samples that can be safely collected even from the smallest animals. This methodology has appeared promising in explaining condition components related to the timing of breeding (Andersson and Gustafsson 1995), physiological changes related to moult and migration (Jenni-Eiermann and Jenni 1994, 1996; Merilä and Svensson 1995) and detection of the physiological costs of reproduction (Gustafsson *et al.* 1994; Hõrak *et al.* 1998). Nevertheless, in spite of this progress, the problems related to selection of optimal research methodology (e.g., Ots *et al.* 1998) and interpretation of test results (e.g., Dufva and Allander 1994; Saino *et al.* 1997 c) continue to persist, suggesting a further need for detailed studies of suitability of different clinical indices for assessment of condition of wild animals.

The aim of this study is to examine the clinical profile of great tits (*Parus major*) in relation to breeding by comparing different, mostly hemato-serological condition indices of individuals captured shortly before egg-laying and in the middle of the nestling period. These two periods are expected to differ in respect of both, environmental and physiological stressors imposed to individuals. Brood rearing exerts considerable energetic demands on the parents (e.g., Drent and Daan 1980), suggesting a stress accompanying increased work load to have a negative impact on the individuals' physiological state. On the other hand, nestlings are reared in the period of greatest animal food abundance which suggests a lack of nutritional constraints. The pre-breeding period, on the contrast, should be more limiting nutritionally, because the insect food supplies have not started to develop yet. At the same time, there are no energetic costs related to brood rearing, suggesting minor health impact due to physical burden. It is, thus, worth investigating how different clinical condition indices respond to these opposite environmental stressors.

In addition to external constraints, pre-breeding and brood-rearing individuals differ in respect to their hormonal status which is known to pose a considerable sex-specific health impact. Particularly, males in the period of mate and territory acquisition/guarding reveal elevated levels of testosterone and corticosterone (e.g., Silverin 1990; Ketterson *et al.* 1991) which, due to their immunosuppressive effects, might lead to deterioration of health state (e.g., Bohus and Koolhaas 1991; Folstad and Karter 1992). We therefore asked whether the seasonal clinical profiles of males and females differ. Our specific expectation was that males are in poorer condition than females in the pre-breeding period.

As complementary measures of individual condition, we employed simple clinical assays including (1) estimates for total and differential leukocyte counts, (2) plasma protein concentrations and profiles, (3) plasma metabolites, (4) hematocrit, (5) body mass, and (6) blood parasitemia. Of these, plasma metabolites, albumin (and sometimes total plasma protein content) are considered indicative of nutritional state (see e.g., Jenni-Eiermann and Jenni 1996; Ots *et al.* 1998), while the leukocyte counts might be informative of inflammatory process, parasite infection and stress. Challenge to humoral immune defence can be detected on the basis of elevated gamma-globulin concentrations and low albumin/globulin ratios (e.g., Kawai 1973). Low values of hematocrit occur in anemia due to parasite infestations or gastro-intestinal infections (Dein 1986) while high values might indicate elevated oxygen consumption accompanying intense work load (Carpenter 1975). Blood parasites are of interest because their relapse in the bloodstream is hormonally triggered (Haberkorn 1968) and the prevalence changes seasonally

(Atkinson and van Riper 1991; Sundberg 1995). Body mass deserves attention as the most commonly used condition index, dealt in almost any study where animals are captured. Our study species was the great tit, a small (ca. 19 g) passerine bird, common throughout the Eurasian continent and belonging to the favourite research objects of avian ecologists.

Methods

The study was conducted during the spring and summer of 1997 in an urban great tit population, breeding in nestboxes in the city of Tartu (58° 22' N, 26° 43' E, human population about 100 000), south-east Estonia. The study area is described by Hõrak *et al.* (1995, 1997). In the pre-breeding period, great tits were captured while roosting in nestboxes during three nights from 26 to 28 April. This period preceded population median onset of egg-laying by 10 days. For individuals captured at both pre-breeding and breeding stages, the shortest interval between the pre-breeding capture and laying of first egg was four days and the longest interval 18 days. Breeding individuals were captured while feeding 8 days old nestlings. At capture, birds were weighed with a Pesola spring balance with a precision of 0.1 g. Blood samples (100–150 µl) for analyses were collected from the tarsal or brachial vein. For identification of blood parasites and leukocytes, a drop of blood was smeared on two individually marked microscope slides, air-dried, fixed in absolute methanol, and stained with azure-eosin. Slides were examined for the presence of blood parasites under 400 × magnification for *Trypanosoma*, microfilaria and *Leucocytozoon*, and under 1000 × with oil immersion for *Haemoproteus* and *Plasmodium*. Individuals were classified as infected when smears were positive for at least one hemoparasite taxon (most common of which was *Haemoproteus* sp.).

After scanning for blood parasites, slides were used to estimate total number and proportion of different types of leukocytes. The proportion of different types of leukocytes was assessed on the basis of an examination of a total of 100 leukocytes under oil immersion. Estimates of the total white blood cell count (WBC) were obtained by counting the number of leukocytes per approximately 10000 erythrocytes. For that purpose, all leukocytes were counted in 100 microscope fields with approximate number of 100 erythrocytes in each. Differential leukocyte counts were obtained by multiplying their proportions with WBC. The repeatabilities of leukocyte counts obtained from repeated scannings of the same blood smear were high ($r = 0.93$, 0.92, 0.87, and 0.86 for heterophile count, heterophile/lymphocyte (H/L) ratio, WBC and lymphocyte count, respectively; all P values < 0.00001; Ots *et al.* 1998). Moreover, measurement errors (estimated as variation coefficients obtained from the repeated sampling of the same individual) of hemato-serological indices used in this study were reasonably low (between 0.06 and 0.25; Ots *et al.* 1998). Analogous methods for estimating leukocyte concentrations have been also used by e.g. Dufva and Allander (1995), Saino *et al.* (1995, 1997 c, 1998), Saino and Møller (1996).

Hematocrit was measured with a digital calliper to the nearest 0.1 mm after 10 minutes of centrifugation of heparinized capillary tubes at 10000 rpm. After determination of hematocrit, plasma was separated from blood cells and stored at –20°C until analysed. 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 densitometrically scanned at 525 ηm wave length. Total plasma protein concentration was determined in a photometric colorimetric test using the biuret method (see e.g., Ibekwe and Ugwunna 1990). Because of difficulties in separating the prealbumin fraction from albumin, summed concentrations for both are reported and termed albumin concentration.

Lipoproteins were determined with a standard agarose gel electrophoresis system Paragon (Beckman) used precisely according to the instructions given by the producer. The lipoproteins were visualised with Sudan Black B stain and quantified by densitometric scanning (Appraise Junior Densitometer, Beckman). For further details and characterization of lipoproteins, see Jenni-Eiermann and Jenni (1992). Plasma metabolites were determined using standard test combinations modified for small amounts of plasma (5µl plasma per determination): quantitative enzymatic tests for triglycerides and glycerol, ß-hydroxy-butyrate (Sigma Diagnostics) and uric acid (Boehringer Mannheim Biochemica). Persons examining blood samples had no information about the phenotypic values of individuals.

None of the parameters examined varied in respect to time of capture in the pre-breeding period. Among the breeding birds, body mass, triglyceride, very low density lipoproteins(VLDL) and uric acid concentration increased during the day. However, this time effect was minute compared to variation between stages of sampling and therefore we did not adjust the data for diurnal pattern when comparing pre-breeding values with these obtained during brood rearing. Our previous research (Ots *et al.* 1998) has revealed that great tits captured at roosting had higher leukocyte (both lymphocyte and heterophile) counts than those captured during daytime. This precludes interpretation of differences of leukocyte counts in relation of the stage of study, as all birds were captured at roost in spring and during the daytime in summer.

Effect of stage of breeding and sex on variation of condition indices was tested in ANOVA models with SAS GLM procedure (SAS Institute 1985), using III type of sums of squares, enabling us to account for all effects of independent variables simultaneously. Because we were interested in sex-specific variation of traits in relation to stage of breeding, each model was tested for the significance of Sex × Stage interaction terms. For a subset of individuals captured in both stages of study, we also present data for inter-individual longitudinal changes between the stages of reproduction, and test them for the significance of change in paired t-tests. For this analysis, we had reasonable sample sizes (n = 5–13) for leukocyte counts, body mass and hematocrit only. Differences in hemoparasite prevalence were tested with χ^2 tests, applying Yates' correction when cell counts were smaller than 10 observations.

All significance levels refer to two-tailed tests. Subscripts used in connection with test statistics refer to degrees of freedom. In ANOVA's, log-transformed values of all parameters except hematocrit, total plasma protein and free fatty acid concentration were used in order to normalise distribution of dependent variables. Correspondence of residuals to normal distribution was checked with Shapiro-Wilk's W-test; in no case was the assumption of normality violated. In the figures, untransformed data are shown in order to present the reference values.

Results

1. Leukocytes

Female great tits had generally higher leukocyte counts than males (Fig. 1 A). Specifically, females had more circulating lymphocytes than males in spring (Fig. 1 B) and more heterophiles than males in summer (Fig. 1 C). WBC and lymphocyte counts of those females that were sampled in both stages decreased during breeding more than corresponding values of males (for females Δ WBC = 30.3, $t_9 = 3.9$, $p = 0.003$; Δ lymphocyte count = 20.0, $t_9 = 4.8$, $p < 0.001$, for males Δ WBC = 20.1, $t_8 = 4.1$, $p = 0.003$; Δ lymphocyte count = 11.1, $t_8 = 3.6$, $p = 0.006$). The result that changes of WBC and lymphocyte counts during breeding were sex-specific was also confirmed by significant Sex × Stage interaction terms in Table 1 A. Heterophile count decreased significantly between sampling episodes only in males ($\Delta = 5.4$, $t_8 = 3.7$, $p = 0.006$) while the difference was not significant in females ($\Delta = 2.7$, $t_9 = 4.4$, $p = 0.4$).

In spring males had higher heterophile/lymphocyte ratios than females, while in summer, the difference was in an opposite direction (Fig. 1 D). Within individuals, H/L ratios increased significantly in females ($\Delta = 0.5$, $t_9 = 3.1$, $p = 0.013$), while there was no statistically significant change in males ($\Delta = 0.1$ $t_8 = 1.0$, $p = 0.3$).

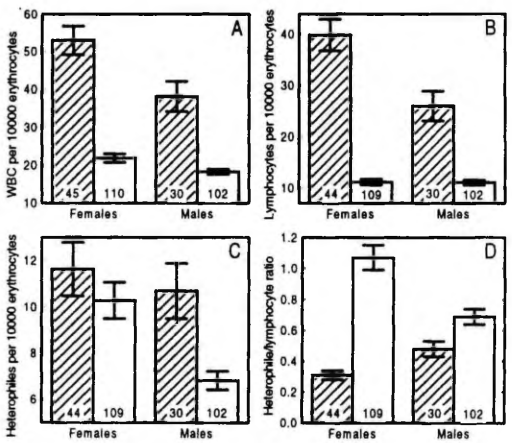

Figure 1. Absolute and differential leukocyte counts of Great Tits in relation to sex and stage of reproduction. In all figures, data are presentes as mean ± SE, hatched bars denote pre-laying period and white bars nestling period. Numbers indicate sample size.

2. Plasma proteins

Concentrations of total plasma protein, albumin and gamma-globulin were significantly higher in the pre-breeding period while the sexes did not differ systematically (Table 1 B, Fig. 2 A–C). Males had generally higher albumin/globulin (Alb/Glo) ratios than females. Besides, males captured in spring had higher Alb/Glo ratio than these

captured in summer, while in the females, the difference was in an opposite direction (Table 1 B, Fig. 2 D).

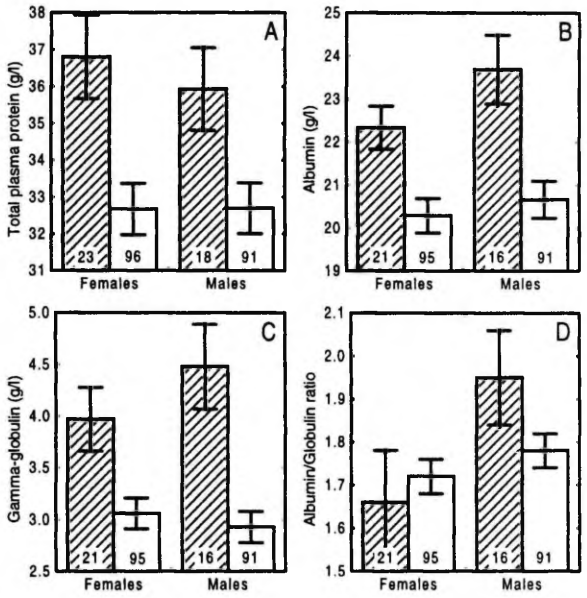

Figure 2. Plasma proteins of Great Tits in relation to sex and stage of reproduction.

3. Plasma metabolites and lipoproteins

Concentrations of all fat metabolites (β-hydroxy-butyrate, glycerol and triglycerides) were higher in the pre-breeding period, while the concentration of uric acid was higher during brood-rearing (Table 1 C, Fig, 3). We could not detect any consistent differences between sexes in respect to plasma metabolite concentrations; neither was any Sex × Stage interaction term in Table 1 C significantly different from zero. Our inability to detect sex-specific differences in ANOVA could possibly be attributed to small sample size, because when pre-breeding birds were analysed separately, males had significantly higher β-hydroxy-butyrate levels than females ($t_{6,3} = 2.6$, $p = 0.028$; Fig. 3 C).

Pre-laying females tended to have higher concentrations of very low density lipoproteins (VLDL) than males and breeding females (Fig. 3 E, Table 1 D). In spring, females had lower percentage of fifth fraction lipoproteins (the quickest moving fraction, representing the free fatty acid, FFA) levels than males, while during the brood rearing, the difference was in the opposite direction (Fig. 3 F), as also indicated by marginally significant Sex × Stage interaction term in Table 1 D.

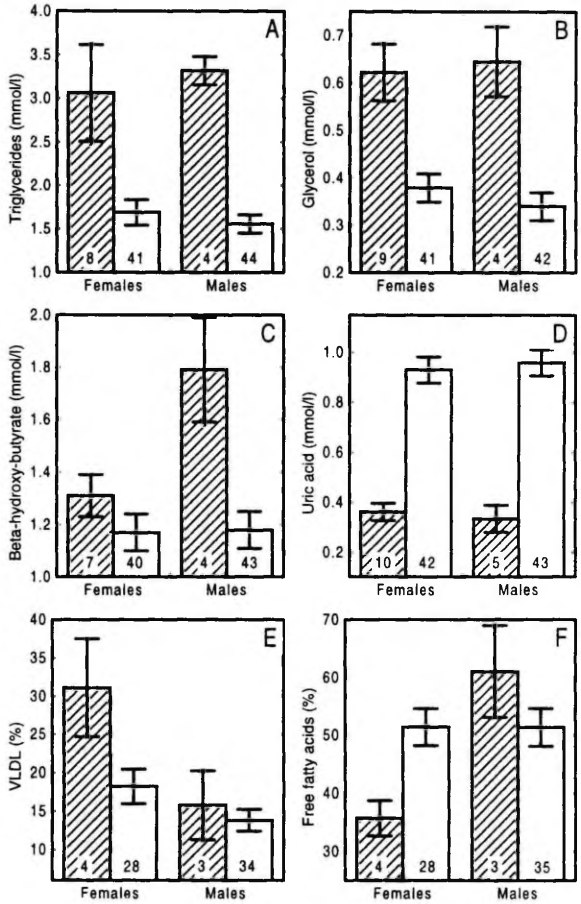

Figure 3. Plasma metabolites of Great Tits in relation to sex and stage of reproduction.

4. Hematocrit

Hematocrit was generally higher in the pre-breeding period (Table 1 E, Fig. 4). Significant Sex × Stage interaction term in Table 1 E was due to the fact that hematocrit of males was lower than that of females during brood-rearing while in the pre-breeding period sexes did not differ. This result was corroborated by comparison of individual hematocrit values of individuals sampled in both stages: in males the hematocrit decreased significantly during breeding ($\Delta = 0.07$, $t_4 = 3.7$, $p = 0.021$) while in females the change was not significantly different from zero ($\Delta = 0.02$, $t_5 = 2.01$, $p = 0.100$).

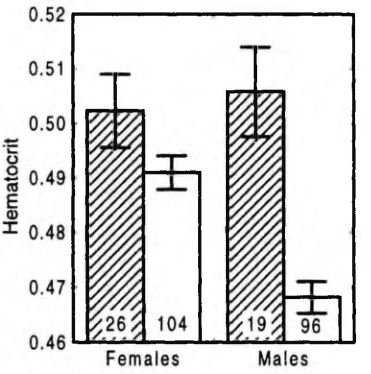

Figure 4. Hematocrits of Great Tits in relation to sex and stage of reproduction.

5. Body mass

Great tits weighed more in the pre-breeding period and males were generally heavier than females (Table 1 E, Fig. 5). Weight loss during the breeding period was similar in both sexes as the Sex × Stage interaction term in Table 1 E was not significant. Comparison of individual body mass profiles revealed the same: both males and females lost weight by nearly 1.5 g between the stages of sampling (males: $\Delta = 1.5$, $t_8 = 4.2$, $p = 0.004$, females: $\Delta = 1.6$, $t_{12} = 3.8$, $p = 0.002$).

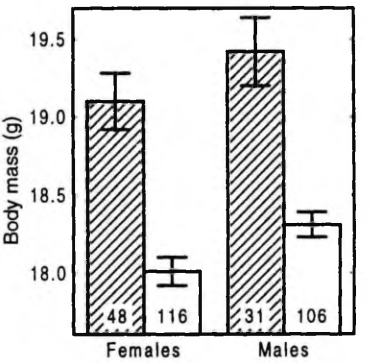

Figure 5. Body mass of Great Tits in relation to sex and stage of reproduction.

6. Blood parasites

Parasites were detected in the blood of 7% (5/75) of great tits, sampled in the pre-breeding period. Hemoparasite prevalence was significantly ($\chi^2_1 = 36.7$, $p < 0.001$) higher in the nestling period when 45% (103/227) of individuals were infected. In nei-

ther of the periods did parasite prevalence differ between sexes (in the prebreeding period 9% of females and 3% of males infected; $\chi^2_1 = 0.20$, p = 0.7, during the brood-rearing 47% of females and 44% of males infected; $\chi^2_1 = 0.17$, p = 0.7).

Table 1. ANOVA's of condition indices of great tits in relation to sex and stage of breeding (pre-laying vs. brood rearing).

A. Leukocytes

Effect	WBC		Lymphocyte count		Heterophile count		H/L ratio	
	F_{DF}	P	F_{DF}	P	F_{DF}	P	F_{DF}	P
Sex	$16.5_{1,283}$	<0.001	$10.8_{1,281}$	0.001	$5.1_{1,281}$	0.024	$0.0_{1,281}$	0.859
Stage	$161.8_{1,283}$	<0.001	$230.1_{1,281}$	<0.001	$10.0_{1,281}$	0.002	$63.0_{1,281}$	<0.001
Sex × Stage	$3.8_{1,283}$	0.052	$14.9_{1,281}$	<0.001	$2.9_{1,281}$	0.088	$19.8_{1,281}$	<0.001

B. Plasma proteins

Effect	Total protein		Albumin		Gamma globulin		Alb/Glo ratio	
	F_{DF}	P	F_{DF}	P	F_{DF}	P	F_{DF}	P
Sex	$0.1_{1,224}$	0.707	$8.2_{1,219}$	0.290	$0.2_{1,219}$	0.659	$8.2_{1,219}$	0.005
Stage	$10.8_{1,224}$	0.001	$14.1_{1,219}$	<0.001	$20.4_{1,219}$	<0.001	$0.0_{1,219}$	0.862
Sex × Stage	$0.2_{1,224}$	0.690	$0.3_{1,219}$	0.577	$0.8_{1,219}$	0.378	$3.8_{1,219}$	0.054

C. Plasma metabolites

Effect	BOH		Triglycerides		Glycerol		Uric acid	
	F_{DF}	P	F_{DF}	P	F_{DF}	P	F_{DF}	P
Sex	$1.9_{1,90}$	0.178	$0.7_{1,93}$	0.401	$0.1_{1,92}$	0.829	$0.0_{1,96}$	0.786
Stage	$7.0_{1,90}$	0.009	$14.9_{1,93}$	<0.001	$15.1_{1,92}$	<0.001	$81.5_{1,96}$	<0.001
Sex × Stage	$1.6_{1,90}$	0.208	$0.2_{1,93}$	0.665	$0.3_{1,92}$	0.590	$0.3_{1,96}$	0.581

D. Lipoproteins

Effect	VLDL		FFA	
	F_{DF}	P	F_{DF}	P
Sex	$3.4_{1,65}$	0.069	$3.1_{1,66}$	0.084
Stage	$3.2_{1,65}$	0.081	$0.2_{1,66}$	0.680
Sex × Stage	$0.8_{1,65}$	0.368	$3.1_{1,66}$	0.083

E. Hematocrit and body mass

Effect	Hematocrit		Weight	
	F_{DF}	P	F_{DF}	P
Sex	$3.5_{1,241}$	0.064	$5.8_{1,298}$	0.017
Stage	$22.3_{1,241}$	<0.001	$68.4_{1,298}$	<0.001
Sex × Stage	$6.4_{1,241}$	0.012	$0.0_{1,298}$	0.967

Discussion

Differences between stages of reproduction

Compared to the brood-rearing stage, great tits captured in the pre-breeding period were on average 1.5 g heavier and had higher concentrations of total plasma protein, albumin and fat metabolites (triglycerides). As all these parameters are indicative of good nutritional state (e.g., Jenni and Jenni-Eiermann 1996; Ots *et al.* 1998), our data suggest that pre-breeding period does not appear nutritionally more limiting for the great tits than nestling period, even though the animal food is much more scarce in early spring. The result that pre-breeding birds had high triglyceride and VLDL levels and low FFA levels is surprising because these birds were sampled during overnight fast during which the plasma lipid concentrations usually decrease (Jenni-Eiermann and Jenni 1994, 1996). Possibly, these high fat levels indicate that the great tits are generally more inclined to fat deposition in the pre-breeding stage than during the brood rearing.

Low concentration of plasma uric acid and high concentrations of the fat catabolites — glycerol and ß-hydroxy-butyrate as found in the pre-laying birds are consistent with typical pattern of overnight fasted birds, which save protein and catabolise their fat reserves, whereas the elevated concentrations of uric acid in the nestling period indicate the feeding state and reflect the catabolism of the dietary protein (Jenni-Eiermann and Jenni 1991).

Heterophile/lymphocyte ratios were generally higher during the breeding period (Fig. 1 D). H/L ratio is a widely used stress index in poultry, known to increase in response to various stressors, including infectious diseases, starvation, and physiological disturbance (e.g., Gross and Siegel 1983; Maxwell 1993). Our results therefore suggest that the brood-rearing period induces greater leukocytic stress response than the pre-breeding period.

Plasma concentrations of gamma-globulin were considerably higher in the pre-breeding period (Fig. 2 C). Gamma-globulin fraction of the plasma includes most of the known antibodies involved in immune response to bacterial, viral, and protozoan diseases (e.g., Kawai 1973). Elevated gamma-globulin levels may therefore signal infection, as shown for brood-rearing great tits infected with blood parasites (Ots and Hõrak in press). Hence, one possible interpretation of elevated gamma-globulin level of pre-breeding birds could be that the pathogenic challenge to the humoral immune system of great tits is more severe in early spring than in summer. However, it has been also shown that an individual's ability to mount a humoral immune response depends on current physiological state. For instance, Saino and Møller (1996) showed that male barn swallows, whose flight costs were increased by experimental tail elongation, failed to increase their gamma-globulin level in response to injection with a novel antigen, while control birds and those with shortened tails demonstrated an increase in gamma globulin level. It might be thus possible, that elevated pre-breeding gamma-globulin levels in our study reflect the trade-off between immune-response and reproductive effort, as pre-breeding birds seem to possess more resources available for immune defence.

Great tits captured in spring had significantly higher hematocrits than these captured in summer. Possibly, this difference was due to lower ambient temperature in pre-breeding period, as high hematocrits in cold periods are expected to reflect increased

oxygen-carrying capacity of blood to sustain higher levels of thermogenesis (see e.g., Dawson and Bortolotti 1997 a).

Prevalence of blood parasites increased from 7% in pre-breeding period to 45% during the brood rearing. It is experimentally shown that relapse of blood parasites can be triggered by stress (e.g., Atkinson and van Riper 1991) or sex hormones (e.g., Haberkorn 1968), which is also likely to explain the seasonal increase of hemoparasite prevalence in our study. Our result that only 7% of individuals were infected in pre-laying period differs from that of Sundberg (1995) who found much earlier relapse of infection prior to breeding in yellowhammers.

Sex differences

As could be expected for a sexually dimorphic species, male great tits were heavier than females in both stages of study. The result that both females and males lost about 1.5 g body mass (7–8% of spring weight) between pre-breeding and breeding period indicates that high female pre-laying body masses in our study were not caused by development of the reproductive system. Most indices of nutritional state (except β-hydroxy-butyrate, VLDL and FFA in spring) did not differ significantly between sexes, suggesting no consistent differences in nutritional condition between male and female great tits. In this respect, our results differ from these of Ots *et al.* (1998), who found marginally higher plasma albumin content in male great tits in the middle of the nestling period. Our result of similarity of females and males in respect to total plasma protein content differs from that of Dawson and Bortolotti (1997 b), who found that in both pre-laying and incubating period, female American kestrels had higher total plasma protein content than males.

Males had remarkably higher β-hydroxy-butyrate and FFA levels than females in spring but not in summer (Fig. 3 C and F). Elevated levels of β-hydroxy-butyrate and FFA are characteristic to fat catabolism during fasting (e.g., Jenni-Eiermann and Jenni 1994), which might suggest that during the overnight fast, males relied more strongly on fat reserves, while females relied more on plasma lipids. It is likely, that the high lipid levels of females tits in the spring were induced by estrogens, which increase significantly before egg laying and decrease thereafter (Sturkie 1986). It should be noted, however, that our sample sizes for the pre-breeding period were small (3–7 females and 3–4 males), which cautions against making any general conclusions on the basis of these data.

Females had generally lower albumin/globulin (Alb/Glo) ratios than males (Fig. 2 D, Table 1 B). Low Alb/Glo ratios result from reduced albumin synthesis in the liver and simultaneous increase of acute phase proteins and immunoglobulins which are typical symptoms of both acute and chronic infectious or inflammatory disease (Kawai 1973, Chaudhary and Sadana 1992). Our data, thus, suggest female great tits reveal generally more symptoms of infectious disease than males. Similar sex difference in Alb/Glo ratios of breeding great tits was also found by Ots *et al.* (1998).

In both stages of study, females had higher total leukocyte counts than males (Fig. 1 A). In spring, this difference was mainly due to higher lymphocyte count of females (Fig. 1 B), while in summer, females had more circulating heterophiles than males (Fig. 1 C) which also lead to greater H/L ratios of breeding females as compared to males (Fig. 1 D). Since peripheral heterophilia is considered as a characteristic symptom

of almost any kind of stress (e.g., Maxwell 1993), inflammation (Parslow 1994), and microbial challenge (Rose *et al.* 1979; Hawkey *et al.* 1985), our results suggest that the health state of breeding females during the brood rearing was worse than that of males. The result, that breeding females had higher heterophile counts and H/L ratios than males, was similar to that found by Ots *et al.* (1998). Possibly, elevated H/L ratios indicate stress caused by intense reproductive effort, since Hõrak *et al.* (1998) found that H/L ratios of female great tits increased in response to experimental brood enlargement. In this context, it is interesting to note that, in the pre-breeding period the H/L ratios of males were higher than these of females (Fig. 1 D). Thus, if H/L ratios of great tits reflect stress in a similar manner as found in poultry studies (e.g., Maxwell 1993), then our data suggest that males were more stressed than females in the prebreeding period, while females were more stressed during the brood rearing. Possibly, this sex difference could relate to additional workload imposed upon females by the brooding activities, in which males do not participate.

The result that males had lower lymphocyte counts than females in spring might be caused by sex-related differences in hormonal profiles. Lower pre-breeding lymphocyte hemoconcentration and higher H/L ratios of males as compared to females might be related to immunosuppressive effects of testosterone and corticosterone, concentrations of which in males are known to peak in the pre-laying period (e.g., Silverin 1990; Ketterson *et al.* 1991). In poultry, corticosterone administration is known to suppress lymphocyte hemoconcentration and increase H/L ratios (e.g., Gross *et al.* 1980; Dohms and Metz 1991).

Hematocrits of male and female great tits did not differ in the pre-breeding period, while during the brood-rearing, females had remarkably higher hematocrits than males. High values of hematocrit are generally indicative of elevated oxygen consumption accompanying high work load (Carpenter 1975). In birds, hematocrits have been shown to increase in response to experimental increase of flight costs in barn swallows (Saino *et al.* 1997 a,b) and brood enlargements in great tits (Hõrak *et al.* 1998). Our results of higher hematocrits of brood-rearing females (see also Ots *et al.* 1998) can therefore be interpreted as indicating higher work load of breeding females as compared to that of males. Analogously to several other studies (reviewed by Dawson and Bortolotti 1997 b), our results do not support the notion of Sturkie (1986) that male birds have higher hematocrits than females as could be expected on the basis of androgenic stimulation and oestrogen-induced suppression of erythropoiesis.

Female and male great tits did not differ in respect of hemoparasite prevalence. The immunocompetence handicap principle (Folstad and Karter 1992) suggests that due to testosterone-induced immunosuppression, males may be more susceptible to parasite infection than females. This clearly was not the case here. In other studies of great tits, Allander and Bennett (1994) failed to detect any sex-related differences in respect to hemoparasite prevalence, while Norris *et al.* (1994) found that females were more frequently infected than males. Studies in other bird species have found higher blood parasite prevalence either in females (Earle and Little 1993; Wiehn *et al.* 1997), or males (Weatherhead and Bennett 1991), or failed to detect any sex differences (e.g., Forbes *et al.* 1994; Odell and Robbins 1994).

Conclusions

In the pre-laying period, scarce of animal food, great tits did not appear to be in worse nutritional condition than during the food-rich nestling period. The metabolic profile pointed to an increased fat metabolism during the pre-laying period, which can partly be explained by fat deposition and partly by the influence of sex hormones. The protein metabolism did not indicate a nutritional stress like food restriction or extreme fasting. Great tits may experience greater stress levels in the brood rearing period than in early spring, as their heterophile/lymphocyte ratios (particularly in females) were generally higher in summer. Pre-laying birds might invest more in humoral immune defence or, alternatively, be exposed to greater immune challenge than breeders, as indicated by elevated gamma-globulin levels in early spring.

In both stages of study, females had generally lower albumin/globulin ratios than males, which might indicate their greater disposition to infectious diseases as compared to males. This result, as well as lack of sex differences in respect to hemoparasite prevalence does not support the immunocompetence handicap principle (Folstad and Karter 1992; Zuk 1996), which suggests that males are more susceptible to infections than females. Possibly, females' generally worse health state (particularly during breeding) was related to their higher work load, as brood-rearing females had higher hematocrits and H/L ratios than males. Both of these indices were previously known to increase in response to brood enlargement (Hõrak *et al.* 1998).

In contrast to breeding period, males seemed to be more stressed than females prior to egg-laying, as indicated by their lower lymphocyte counts and higher H/L ratios as compared to females at the same time. This result would be expected if pre-laying male great tits had elevated levels of immunosuppressive testosterone and corticosterone as shown for other passerine species (e.g., Silverin 1990; Ketterson *et al.* 1991).

Taken together, our data suggest that the clinical profiles of male and female great tits differ in relation to stage of reproduction, males being more stressed than females prior to egg-laying, and females being in worse condition than males while feeding nestlings. The first result indirectly supports the notion of hormonal impact for life histories (Ketterson and Nolan 1992), while the second points to the inter-sexual conflict over brood rearing.

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TECHNICAL REPORT

Haematological health state indices of reproducing Great Tits: methodology and sources of natural variation

I. OTS,*† A. MURUMÄGI* AND P. HÕRAK‡†§

**Department of Zoology and Hydrobiology, Tartu University, Riia 181, EE2400 Tartu, Estonia, †Department of Zoology, Uppsala University, Villavägen 9, S-75236 Uppsala, Sweden, and ‡Estonian Institute of Zoology and Botany, Riia 181, EE2400 Tartu, Estonia*

Summary

1. A cornerstone concept of ecological immunology is that immune function, interacting with various aspects of individual health state, plays a central role in the life-history trade-offs between conflicting demands of survival and reproduction. In order to develop this research, more knowledge about the applicability and usefulness of different health state assays is needed.

2. Eleven, mostly hemato-serological, health state indices are described and their suitability for sensing the condition of breeding Great Tits (*Parus major* L.) in terms of measurement precision, constancy in time, diurnal variation, and sex- and site-related differences, is examined.

3. Measurement errors for the plasma albumin content, residual body mass, heterophile/lymphocyte ratio and total plasma protein content were relatively small compared with the total variation, suggesting these indices to be most adequate for ecological research. Measurement precision was lowest for the heterophile count and 'buffy coat' layer height (relative amount of leucocytes in total blood volume). Buffy coat layer height correlated weakly ($r=0{\cdot}21$) with total leucocyte count estimated from blood smears and therefore appeared inappropriate for estimation of the leucocyte number.

4. Body mass (residual in respect to size) and intensity of *Haemoproteus* blood parasite infection were the least variable state indices during the nestling period (for both, the correlation between the values measured on the 8th and 15th days of the nestling period = 0·71). Haematocrit, heterophile count and albumin/globulin ratio showed no individual constancy across the nestling period, while other traits revealed moderate but statistically significant correlations between 8th- and 15th-day values.

5. Leucocyte (both lymphocyte and heterophile) counts were higher among females captured at night compared with those captured during the day.

6. Females had higher intensities of *Haemoproteus* infection, higher heterophile counts and higher heterophile/lymphocyte ratios than males. Contrary to published information, females had higher haematocrits than males.

7. Haematocrit values in both sexes, as well as total plasma protein and albumin concentrations in males, differed significantly between Great Tits breeding in urban habitat and rural woodlands, respectively.

Key-words: Leucocytes, measurement precision, *Parus major*, plasma proteins

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Introduction

The recent understanding that immune function may play a crucial role in the mechanisms behind sexual selection (Hamilton & Zuk 1982), life-history trade-offs (Gustafsson *et al.* 1994), animal population regulation (Lochmiller & Dabbert 1993; Lochmiller 1996) and state-dependent reproductive decisions (McNamara & Houston 1996) has given rise to a new discipline labelled as ecological immunology by Sheldon and Verhulst (1996). Because immune function interacts with the general health state of an organism and

§ Correspondence: Estonian Institute of Zoology and Botany, Riia 181, EE2400 Tartu, Estonia; e-mail: horak@zbi.ee

competes for the resources that can be allocated to other activities, immunological research may offer a powerful tool for explaining how reproductive effort links to reproductive costs. No less importantly, it has the potential of promoting the development of state-dependent life-history theory by explaining why individuals differ from each other with respect to their reproductive decisions. On these grounds, a burst of immunological research in the context of evolutionary animal ecology can be predicted.

An important issue regarding the application of various immunoassays to the study of natural animal populations is the choice of optimal research methodology. Different health state indices measure different aspects of individual condition, and may also reflect health disorders of different duration. Moreover, there are several sources of variation not directly related to the life-history components under examination, such as diurnal variations and differences due to sex and habitat, awareness of which is of crucial importance for distinguishing between environmental noise and experimental effect. Different methods also require different amounts of financial and laboratory power, and, finally, are subjected to different measurement errors. For choosing appropriate research methods, consideration of all these aspects is of substantial importance.

The aim of this study is to describe the sources of natural variation of 11 (mostly hemato-serological) health state indices in reproducing Great Tits (*Parus major*). This species, like several other small hole-nesting passerines, belongs to the favourite research objects of animal ecologists, which makes the knowledge about the applicability of different assays for describing their health condition important. However, no detailed information about the suitability of different haematological methods for sensing the health state of wild bird species has been reported yet. In this paper, we address questions about measurement precision, constancy in time, diurnal variations, and sex- and habitat-related differences of health state indices. A companion paper (Hõrak, Ots & Murumägi 1998) will describe the effects of brood size manipulation on the same parameters.

Health state indices examined in this study can be divided into the following five categories.

PLASMA PROTEINS

Plasma proteins provide various transport and immune functions, and exert a colloidal osmotic pressure that aids in the preservation of blood volume and helps maintain the blood pH within a narrow range. Decrease in total plasma protein concentration (hypoproteinaemia) is seen in almost all diseases but especially in the case of malnutrition. Physical exercise or water shortage, in turn, results in an increase of total plasma protein level because of haemoconcentration (Kawai 1973). Of the specific plasma proteins, concentration will be placed on albumin and total globulin levels.

Albumin is the highest concentration single protein in the normal plasma and serves as an amino acid pool for protein synthesis, acts as a carrier of many metabolites and can be used as an energy source in the case of exhaustion of glycogen and lipid reserves. Albumin is potentially an interesting health state index for ecological animal studies because its relative or absolute decrease in the serum (hypoalbuminaemia) is a common symptom of almost every pathological state. Among the main causes of hypoalbuminaemia are malnutrition, liver disorders and inflammation, especially acute phase response (Kawai 1973; Rodgers 1994).

The globulin fraction of plasma proteins consists of acute phase proteins, which assist an organism to survive the period immediately following injury, and immunoglobulins (Ig) that bind antigens. Increased Ig level is observed in response to exogenous or endogenous antigenic simulations, including chronic infections. Corticosteroids (stress hormones) are known to reduce the plasma Ig level by suppressing lymphocyte activity (Kawai 1973).

In both acute disease and chronic infections or inflammatory disease, diseased individuals reveal higher total globulin concentration and lower albumin/globulin (Alb/Glo) ratios than in healthy individuals (Kawai 1973).

TOTAL AND DIFFERENTIAL LEUCOCYTE COUNTS

Leucocytes form the basis of the immune system of organism, and their main function is its protection against various pathogenic antigens. Elevated leucocyte number (leucocytosis) is symptomatic of stress syndrome and inflammatory processes. Leucocytosis is in most cases caused by a rising number of heterophils in the peripheral blood (e.g. Dein 1986).

Heterophils (avian counterparts of mammalian neutrophils) are phagocytosing cells that enter the tissues during the inflammatory response. They are non-specific immune cells, and their lysis during the inflammatory response may be harmful to host tissues (Parslow 1994).

Unlike the heterophilic response, the immune response by lymphocytes is always highly specific and causes little or no damage to normal host tissue. Two main cell types are involved in the specific immune response: thymus-derived T lymphocytes which are concerned with immune regulation and antigen elimination, and B lymphocytes (originating from the bursa of Fabricius) which synthesize and secrete immunoglobulins. Decreased lymphocyte number signals immunosuppressions with a concomitant increase in susceptibility to viral infections (Siegel 1985; Fitzgerald 1988).

An index comprising the relative abundance of both lymphocytes and heterophils is the heterophile/

lymphocyte (H/L) ratio, which is a widely used stress estimator in poultry (Gross & Siegel 1983; Maxwell 1993). H/L ratio is known to increase in response to various stressors, including infectious diseases, starvation and psychological disturbance.

BLOOD PARASITAEMIA

Most blood parasites are vector-transmitted protozoans living in tissues and in the peripheral blood of their hosts, consuming a variety of host metabolites and haemoglobin. Blood parasitaemia is of potential interest as an indicator of individual health state because of its expected association with immunocompetence. In this study, we use the intensity of *Haemoproteus* blood parasite infection as an index of parasitaemia.

HAEMATOCRIT

Haematocrit or packed cell volume (PCV) measures the relative amount of red blood cells in total blood volume and reflects the extent and efficiency of oxygen uptake and transfer to tissues. PCV values have been regarded as proportional to metabolic activity during a period of days to weeks preceding blood sampling (Carpenter 1975). Additionally, low values of PCV (anaemia) are indicative of bacterial infections and gastrointestinal disorders, including parasitism and haemorrhage (Dein 1986), or may reflect nutritional deficiencies of minerals like iron or copper (Sturkie & Griminger 1986).

RESIDUAL BODY MASS

Body mass is affected by a multitude of components of individual state, which complicates the interpretation of its variation. However, it deserves attention as a state index owing to the ease of its measurement and wide use in almost any study dealing with captured animals. To be separated from structural size, it has to be expressed as a residual with respect to size.

Methods

Data were collected during the breeding period of 1996 in two neighbouring (urban and rural) Great Tit populations breeding in nestboxes in and near the city of Tartu, south-east Estonia (58 ° 22 ', 26 ° 43 'E). Adult Great Tits were captured on their nests when nestlings were 8-days-old; a subset of individuals were recaptured on the 15th day of the nestling period for the repeated measurement of state indices. Birds were weighed with a Pesola spring balance with a precision of 0·1 g and their tarsi measured with a sliding caliper to the nearest 0·1 mm by the same person (P.H.). To reduce the influence of structural size and diurnal variation, body mass was expressed as the residual from a multiple linear regression of mass on cubed tarsus length and weighing time (measured from sunrise).

Blood samples (100–150 µl) for haematological measurements were taken from the tarsal or brachial vein. For identification of blood parasites and leucocytes, a drop of blood was smeared on two individually marked microscope slides, air-dried, fixed in absolute methanol and stained with azure-eosin. Slides were examined for the presence and abundance of blood parasites under 400× magnification for *Trypanosoma*, Microfilaria and *Leucocytozoon*, and under 1000× with oil immersion for *Haemoproteus* and *Plasmodium*. Scanning of a single slide for parasites took approximately 15 min. Because *Haemoproteus* sp. was the only common blood parasite, occurring in 47% (88/188) of the individuals, our examination of intensity of parasitaemia was confined to this genus.

After scanning for blood parasites, slides were used to estimate total number and proportion of different types of leucocytes. The proportion of different types of leucocytes was assessed on the basis of an examination of a total of 100 leucocytes under oil immersion. Estimates of the total white blood cell count (WBC) and intensity of parasitaemia were obtained by counting the number of leucocytes or parasites per approximately 10 000 erythrocytes. Differential leucocyte counts were obtained by multiplying their proportions with WBC. In the analyses, only data for lymphocytes and heterophils as the most numerous immune cells are used. Scanning of a single slide for leucocyte proportions took approximately 15 min and for the WBC 10 min.

Because WBC is the most widely used and most important haematological test, but at the same time the most difficult one to measure (e.g. Gustafsson *et al.* 1994), some authors have applied alternative methods for its estimation. For comparative purposes, an additional WBC estimate was therefore used, obtained by measuring the height of the 'buffy coat' layer in a haematocrit capillary tube, as used by Gustafsson *et al.* (1994) and Merilä & Svensson (1995). In this case, the blood was sampled into heparinized haematocrit capillary tubes, centrifuged for 10 min at 10 000 r.p.m. after which the height of the leucocyte layer was immediately measured. After measuring the buffy coat, the same capillaries were used for measuring the haematocrit. The time lag between blood sampling and processing was usually 3–10 h, and never more than 12 h. Meanwhile, the capillary tubes, sealed with wax at the bottom end, were left to stand in a vertical position. The measurements were made with a digital caliper to the nearest 0·05 mm under a 15× magnification glass fitted with a light source. The persons examining blood samples (I.O. for Hematozoa, haematocrit, 'buffy coat' and plasma, and A.M. for leucocytes) had no information about the individual birds except ring number.

Plasma samples for protein electrophoresis were obtained by centrifuging blood for 10 min at

3000 r.p.m., after which the plasma was stored at −20 °C until analysed. 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 densitometrically scanned at 525 nm wavelength. Total plasma protein concentration was determined in a photometric colorimetric test using the Biuret method. Because of difficulties of separating the prealbumin fraction from albumin, summed concentrations for both are reported and termed albumin concentration.

In order to assess the counting precision of leucocytes and parasites, a portion of the blood smears were scanned more than once, and the repeatabilities of the counts were calculated as intraclass correlation coefficients from one-way ANOVA according to Lessels & Boag (1987). To obtain more estimates for measurement precision, blood from some individuals was sampled several (3–10) times during the same capturing episode. Coefficients of variation (CV) among these measurements were considered as estimates of measurement error. These estimates were compared with the CVs within the whole sample (Fig. 1).

For calculation of repeatabilities, CVs and correlation coefficients, log-transformed values of all parameters except haematocrit and residual body mass were used in order to obtain normal distribution of data values. Untransformed values were used for haematocrit and residual mass because these were normally distributed.

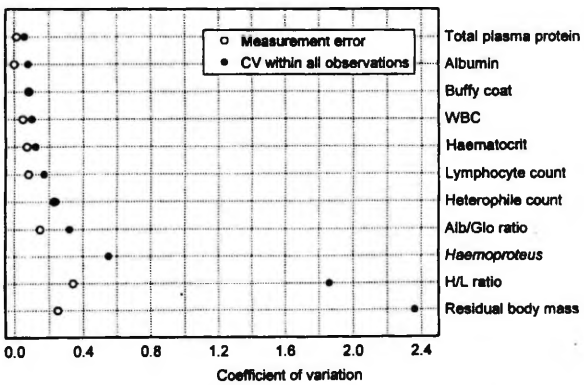

Fig. 1. Comparison of general variability and measurement errors of 11 physiological state indices. General variability is defined as a coefficient of variation (CV) among all sampled individuals ($n = 88 \ldots 211$). Measurement error is defined as CV among different estimates obtained from the same individual during the same sampling episode. Sample sizes are 2 (individuals) × 10 (samplings) for leucocyte counts, 5×2 ... 3 for total plasma protein, 1×3 for specific plasma proteins, 3×3 ... 5 for body mass, 3×6 ... 8 for haematocrit and buffy coat. Overlapping dots suggest low accuracy of assessment as the measurement precision is proportional to the ratio of general variation to measurement error. All trait values except haematocrit and body mass are log-transformed. The cell and parasite counts are estimated as number per 10 000 erythrocytes and plasma protein concentrations measured in $g\,l^{-1}$.

Some individuals were subjected to brood size manipulations within the range of normal brood size. These birds were not excluded from the comparisons of state indices with respect to sex, habitat and sampling time, in order to increase the sample size. Inclusion of manipulated individuals did not bias the results as between-group differences found in pairwise calculations remain significant in ANOVAS accounting for the manipulation and other multiple factor effects (Hõrak *et al.* 1998).

All significance levels refer to two-tailed tests. Data values are reported as mean ± SD. Subscripts used in connection with *t*-tests and Mann–Whitney *U*-tests refer to sample sizes. Only data from genuine first clutches were used. Females incubating in empty nests were excluded from all analyses because they were known to differ from the 'normal' birds in respect of haematological parameters (Ots & Hõrak 1996).

Results

GENERAL VARIATION AND MEASUREMENT PRECISION

Among the 11 state indices studied, residual body mass and H/L ratio revealed the greatest variation at the population level (CV = 1·9 and 2·4; Fig. 1). For all other traits, the coefficient of total variation was less than 0·6, total plasma protein and albumin being least variable (CV = 0·06 and 0·08).

Comparison of measurement errors (variation coefficients, obtained from the repeated sampling of the same individual) and total variation coefficients suggested that the measurement precision was highest for the plasma albumin content, residual body mass, H/L ratio and total plasma protein content (corresponding measurement error/total CV ratios equal to 1%, 11%, 18% and 24%). For albumin/globulin ratio, lymphocyte count and haematocrit the measurement errors comprised 46–48% and for the WBC 58% of the coefficient of variation within all observations. For the buffy coat layer height and heterophile count the measurement error was in the same magnitude of total variation (respectively 91% and 96% of the total CV; see also Fig. 1).

Repeatabilities for leucocyte and parasite counts obtained from repeated scannings of the same blood smears were highly significant (Table 1).

The correlation between two independent estimates of total leucocyte number, namely the buffy coat layer and the WBC count, was remarkably low ($r_s = 0{\cdot}21$, $P = 0{\cdot}010$, $n = 137$). The first-mentioned variable correlated positively with total plasma protein concentration ($r_s = 0{\cdot}33$, $P = 0{\cdot}003$, $n = 79$). This correlation was due to the close relationship between buffy coat height and total plasma globulin concentration ($r_s = 0{\cdot}45$, $P < 0{\cdot}001$, $n = 79$), while the concentration of plasma albumin was not related to buffy coat height ($r_s = 0{\cdot}11$, $P = 0{\cdot}318$, $n = 79$).

CONSTANCY OF STATE INDICES DURING THE NESTLING PERIOD

According to Fig. 2, the least variable traits during the nestling period were body mass and intensity of *Haemoproteus* infection ($r = 0.71$ for both). Lymphocyte count, plasma albumin concentration, WBC, buffy coat, H/L ratio and total plasma protein concentration on the 15th day of the nestling period were moderately but significantly correlated with the corresponding 8th-day values ($r = 0.49$, 0.42, 0.41, 0.39, 0.33 and 0.30, respectively). Haematocrit, heterophile count and Alb/Glo ratio, by contrast, showed no individual constancy during the nestling period.

Table 1. Repeatability (r) of parasite and leucocyte counts, repeatedly measured from the same slides. All P-values <0.00001. Traits are presented in the descending order of r values

Trait	r	F ratio (DF)
Haemoproteus intensity	0.96	48.22 (9 10)
Heterophile count	0.93	27.76 (45 54)
H/L ratio	0.92	27.21 (18 24)
WBC	0.87	14.47 (45 54)
Lymphocyte count	0.86	13.65 (45 54)

Table 2. State indices of breeding female Great Tits captured while roosting in the nestboxes ('Night') and feeding nestlings ('Day'). Leucocyte and parasite counts are expressed as number/10 000 erythrocytes

Trait	Night Mean ± SD (n)	Day Mean ± SD (n)	P diff.
Total plasma protein (g/l)	33.6 ± 5.0 (10)	33.5 ± 9.7 (67)	0.525[a]
Albumin (g/l)	20.9 ± 4.7 (7)	20.4 ± 4.7 (46)	0.799[a]
Albumin/globulin ratio	1.7 ± 0.5 (7)	1.8 ± 0.6 (46)	0.358[a]
WBC	96.6 ± 24.4 (11)	50.7 ± 23.6 (76)	<0.001[a]
Buffy coat height	0.010 ± 0.001 (6)	0.007 ± 0.003 (63)	0.007[a]
Lymphocyte count	58.4 ± 20.4 (11)	26.8 ± 15.0 (76)	<0.001[a]
Heterophile count	37.8 ± 17.6 (11)	23.7 ± 14.5 (76)	0.011[a]
H/L ratio	0.72 ± 0.42 (11)	1.12 ± 1.02 (76)	0.174[a]
Haemoproteus intensity	19.7 ± 15.6 (7)	101.0 ± 376.0 (29)	0.924[a]
Haematocrit	0.56 ± 0.04 (6)	0.54 ± 0.05 (65)	0.216[b]
Residual body mass	−0.41 ± 0.69 (11)	−0.51 ± 0.84 (77)	0.703[b]

[a]Mann–Whitney U-test, [b]t-test.

Table 3. Comparison of state indices of female and male Great Tits. Roosting females are excluded from comparisons concerning leucocyte counts, as these parameters revealed diurnal variation. Leucocyte and parasite counts are expressed as number/10 000 erythrocytes

Trait	Females Mean ± SD (n)	Males Mean ± SD (n)	P diff.
Total plasma protein (g/l)	33.5 ± 9.1 (78)	34.2 ± 8.1 (65)	0.511[a]
Albumin (g/l)	20.5 ± 4.6 (53)	21.8 ± 4.4 (49)	0.053[a]
Albumin/globulin ratio	1.8 ± 0.6 (54)	1.9 ± 0.4 (58)	0.072[a]
WBC	50.9 ± 23.5 (77)	49.7 ± 22.8 (82)	0.809[a]
Buffy coat height	0.007 ± 0.003 (64)	0.008 ± 0.005 (58)	0.778[a]
Lymphocyte count	26.9 ± 14.9 (77)	30.4 ± 17.2 (82)	0.131[a]
Heterophile count	23.8 ± 14.5 (77)	18.7 ± 15.6 (82)	0.014[a]
H/L ratio	1.07 ± 0.97 (83)	0.72 ± 0.56 (83)	0.001[a]
Haemoproteus intensity	82.6 ± 333.4 (37)	61.6 ± 64.1 (42)	0.006[a]
Haematocrit	0.54 ± 0.05 (72)	0.51 ± 0.05 (62)	0.001[b]
Residual body mass	−0.49 ± 0.82 (88)	−0.40 ± 0.82 (82)	0.543[b]

[a]Mann–Whitney U-test, [b]t-test.

DIURNAL CHANGES

According to Table 2, in the period of feeding nestlings, the females that were captured at their nests at night had higher total leucocyte count and buffy coat height, as compared with females captured at daytime. This difference was due to an elevated number of both heterophils and lymphocytes. Other state indices did not differ among females captured while feeding and roosting, respectively.

SEX DIFFERENCES

Females had significantly higher haematocrits and H/L ratios than males (Table 3). The difference in H/L ratio was due to a higher heterophile count in the females while the lymphocyte counts did not differ between sexes. Females also suffered a higher intensity of *Haemoproteus* infection than males. Plasma albumin level and Alb/Glo ratio revealed a non-significant tendency of higher values in males.

DIFFERENCES AMONG STUDY SITES

Urban male Great Tits had higher concentrations of total plasma protein and albumin than rural males ($35.6 \pm 8.5\ g\,l^{-1}$ *vs* $31.6 \pm 6.7\ g\,l^{-1}$; $z_{42,23} = 2.18$, $P = 0.029$ for total protein and $22.7 \pm 4.0\ g\,l^{-1}$ (*vs* $20.2 \pm 4.7\ g\,l^{-1}$; $z_{32,17} = 2.52$, $P = 0.012$ for albumin; Mann–Whitney U-test). Both female and male rural Great Tits had higher haematocrits than their urban conspecifics (0.56 ± 0.05 *vs* 0.53 ± 0.04; $t_{25,47} = 3.36$; $P = 0.001$ for females and 0.54 ± 0.05 *vs* 0.49 ± 0.05; $t_{24,38} = 4.66$; $P < 0.001$ for males respectively).

Discussion

SOURCES OF ERROR

Measurement errors for the plasma albumin content, residual body mass, H/L ratio and total plasma protein content were relatively small compared with the total variation, suggesting these indices to be most adequate for ecological research purposes. For the buffy coat layer and heterophile count, the magnitudes of measurement errors were very close to the estimates of total variation, implying a relatively low precision of measurement. The result that heterophile count had high repeatability (Table 1) but also relatively high measurement errors (Fig. 1) indicates that the error resulted from incorrect assessment of erythrocyte number in different smears obtained from the same individual. The most likely reason for this

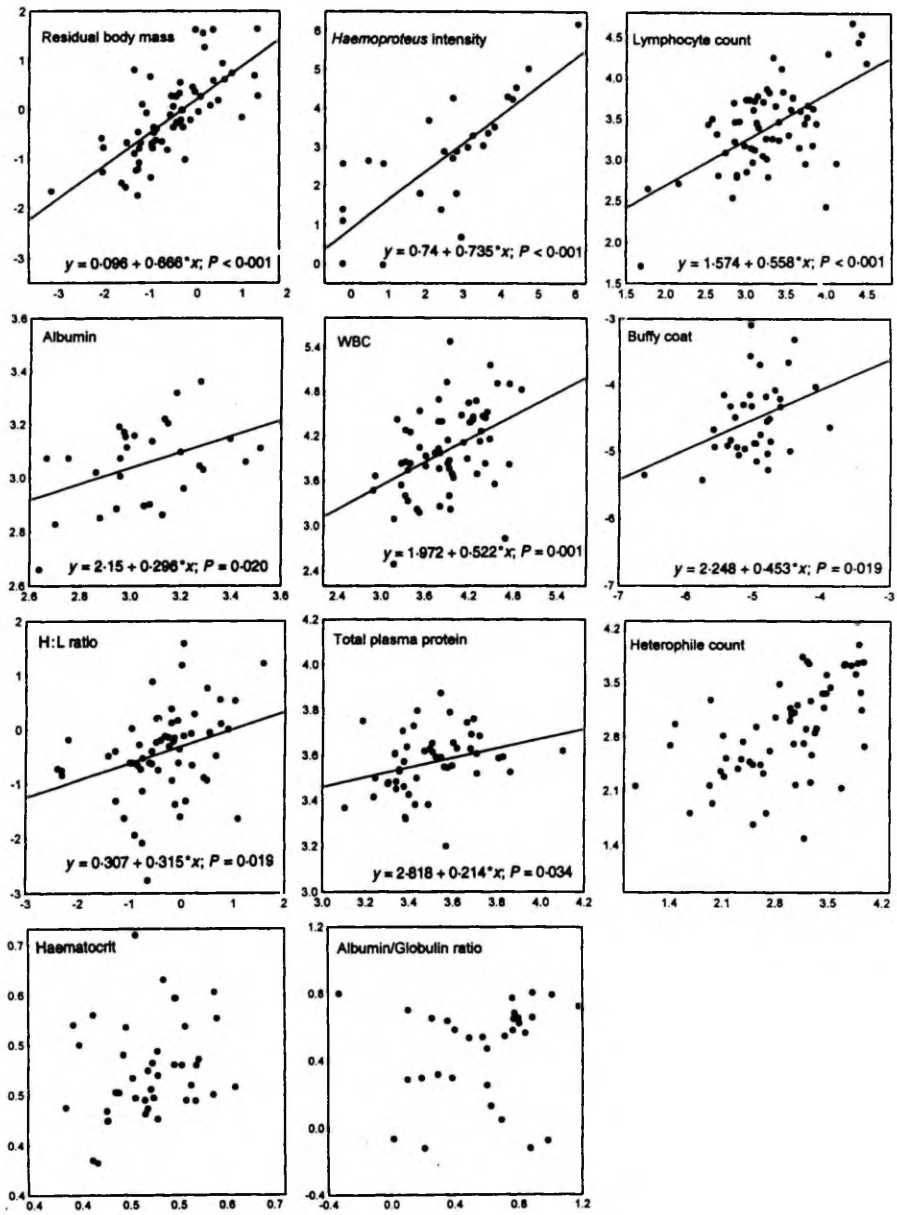

Fig. 2. Relationship between individual state index values at 8th (*x*-axis) and 15th (*y*-axis) days of the nestling period. All trait values except haematocrit and body mass are log-transformed.

flaw was unequal thickness of the smears. Such errors cannot be detected on the basis of repeatabilities estimated by blind scoring of the same smears. Notably, our measurement errors for lymphocyte (CV = 0·08) and heterophile (CV = 0·23) counts were very similar to those reported by Olson (1937) for phyloxine-stained smears (CV = 0·09 and 0·30 respectively; cited in Sturkie & Griminger 1986), which indicates that obtaining correct estimates for heterophile counts from blood smears might be a general problem.

Surprisingly, only a weak relationship ($r_s = 0{\cdot}21$) was found between two estimates of white blood cells count (buffy coat layer and WBC).

CONSTANCY OF HEALTH STATE INDICES IN TIME

An avian ecologist often encounters a major problem when wishing to use the state indices measured during some stage of breeding cycle, as a reference to some persistent component of individual condition. It is therefore important to know for how long a time period a certain measurement is valid. Our results suggest that residual body mass and intensity of *Haemoproteus* infection are relatively stable condition indices, at least within periods of 1-week duration during brood rearing. However, no long-term extrapolations concerning an individual's state should be made on the basis of haematocrit, heterophile count and albumin/globulin ratio. The relative constancy of residual body mass indicates that its ample variation (Fig. 1) is not caused by short-term changes in individual condition and, therefore, may relate to some permanent state differences among individuals.

DIURNAL VARIATION

All four parameters associated with leucocyte counts revealed significant diurnal variation. Females sampled at night had higher numbers of both heterophils and lymphocytes, leading to a higher total leucocyte count and buffy coat layer. Similar diel patterns have been observed in the peripheral blood of mammals (Northern, Rutter & Peterson 1994; Yashiki, Kusunose & Takagi 1995). Diurnal variation of various leucocyte subsets have been reported in pigeons (Shaw 1933) and domestic fowl (Maxwell 1981; Kondo *et al.* 1992). A possible reason for high nocturnal cell counts might be allocation of metabolic energy, which otherwise would be used for daily activities, to cell proliferation (Ricklefs 1979; Starck 1996). Alternatively, lower daytime leucocyte numbers might be caused by movement of the leucocytes out of the blood stream during the day. Circadian variation in leucocyte number should certainly be taken into account in studies using blood samples from roosting females. Notably, no diurnal variation was detected in plasma protein; levels of which are supposed to decrease during sleep (e.g. Kawai 1973).

SEX DIFFERENCES

Four of 11 parameters studied showed significant differences between sexes. Females had more heterophils (which inevitably led to higher H/L ratio); also their haematocrits and intensities of parasitaemia were higher. Males, in turn, had (albeit not significantly) higher plasma albumin levels and albumin/globulin ratios. These differences can possibly be ascribed to the different endocrinology of sexes; however, the possibility that members of one sex systematically work harder and are subjected to more severe stress cannot be excluded either. In this context it is striking that all sex differences among state indices suggest that females were in worse condition and more stressed than males. Concerning parasites, however, it should be noted that higher parasitaemia in females might not be typical since no sex difference in respect to *Haemoproteus* infection was observed in our study area in the previous year (Ots 1996).

Our observation that females had higher haematocrits than males is rather exceptional, in view of the general tendency of higher male haematocrits as reported in previous studies (see Prinzinger & Misovic 1994) and usually explained with reference to the sex steroids (androgens stimulate erythrocyte synthesis and oestrogens inhibit it). Notably, however, studies comparing haematocrits of females and males in the nestling period have not detected sex differences (Silverin 1981; Morton 1994). The tendency of higher albumin concentration in males went in the expected direction as male steroid hormones are known to increase plasma albumin levels. Gayathri and Hedge (1994) found that non-breeding male pigeons had higher albumin levels than females; during the breeding period, however, this difference levelled off.

SITE DIFFERENCES

The higher total protein and albumin level of urban males suggests that urban males are in better general health condition than rural birds. The higher haematocrits of rural birds can have two possible explanations. First, because urban birds are exposed to lead pollution by traffic exhaust fumes, their lower haematocrits might be caused by lead-induced suppression of the haemopoiesis (see, for example, Kostelecka-Myrcha, Zukowski & Oksiejczuk 1997). Second, because the rural Great Tits in general seem to make a greater reproductive effort (Hõrak 1995; Ots & Hõrak 1996), their higher haematocrits can be associated with exercise-induced polycythaemia due to intense work load.

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Indrek Ots · Peeter Hõrak

Health impact of blood parasites in breeding great tits

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Abstract Hypotheses of hemoparasite-mediated sexual selection and reproductive costs rely on the assumption that avian blood parasite infections are harmful to their hosts. To test the validity of this assumption, we examined the health impact of *Haemoproteus* blood parasites on their great tit (*Parus major*) host. We hypothesised that if blood parasites impose any serious health impact on their avian hosts, then infected individuals must differ from uninfected ones in respect to hemato-serological general health and immune parameters. A 3-year study of two great tit populations, breeding in contrasting (urban and rural) habitats in south-east Estonia, revealed that *Haemoproteus* blood parasites affected the health state of their avian hosts. Infected individuals had elevated lymphocyte hemoconcentration and plasma gamma-globulin levels, indicating that both cell-mediated and humoral immune response mechanisms are involved in host defence. The effect of parasites on cell-mediated immunity was both age- and sex-specific, as infection status affected peripheral blood lymphocyte counts only in males, and among these, the magnitude of response was greater in old individuals than yearlings. Heterophile hemoconcentration and plasma albumin levels were not affected by infection status, suggesting that blood stages of *Haemoproteus* infection do not cause a severe inflammatory response. Parasitism was not related to hematocrit values, indicating that *Haemoproteus* infection does not cause anemia. In two years, infected individuals were heavier than uninfected ones in the urban but not in the rural study area. This suggests, that under certain circumstances (possibly related to reproductive tactics), breeding great tits may avoid losing body mass in order to save resources for an anti-parasite immune response.

Key words Immune response · Lymphocytes · Gamma-globulin · *Parus major* · *Haemoproteus*

I. Ots
Institute of Zoology and Hydrobiology,
Tartu University, Riia 181, EE2400 Tartu, Estonia

P. Hõrak (✉)
Estonian Institute of Zoology and Botany,
Riia 181, EE2400 Tartu, Estonia
e-mail: horak@zbi.ee; Fax: +3727-383013

Introduction

Avian blood parasites (Haematozoa) were discovered by V. Danilewsky in 1884 and are now known to occur among most of the nearly 4000 bird species examined so far (Atkinson and van Riper 1991). During the past decade, ecologists have become increasingly interested in their effect on host behaviour and evolution. In particular, much research was stimulated by Hamilton and Zuk (1982) who proposed that colorful plumages have evolved as a signal indicating good health due to successful parasite resistance. Additionally, blood parasites have captured attention in the context of studies of reproductive costs. Here, it is important that the immune defence of an individual can be traded against resources invested in reproduction; and increased parasitemias accompanying experimentally increased parental work load have been interpreted as revealing a physiological cost of reproduction (e.g., Norris et al. 1994; Richner et al. 1995; Allander 1997).

The crucial point in testing both the Hamilton-Zuk hypothesis and various other hypotheses of the importance of immune-mediated reproductive costs is the question of the validity of the underlying assumption that blood parasite infections are harmful to their hosts. This is not self-evident, since an alternative view suggests that parasite resistance is sensitive to manipulation of reproductive effort just because it can be sacrificed at little cost (Sheldon and Verhulst 1996). In this case, the fact that effects of brood manipulation on hemoparasite infection are readily detected can be interpreted as reflecting the relative unimportance of blood parasites in determining a host's residual reproductive value.

Distinguishing between these alternative explanations requires experimental manipulation of hemoparasite infection. However, because of the vector-transmitted nature of blood parasite infections, such tests would be extremely complicated to perform in field conditions.

Apart from methodological issues, the assumption that blood parasites are costly to their hosts may be challenged on the basis of studies testing for the relationship between haematozoan infection and mortality in wild bird populations. Most of these have revealed no negative impact of blood parasite infection on individual survival rates (12 studies reviewed by Bennett et al. 1993; Davidar and Morton 1993; Dale et al. 1996; Siikamäki et al. 1997; but see Richner et al. 1995).

In view of this knowledge, the question whether blood parasites are at all suitable for testing hypotheses in evolutionary avian ecology seems to be justified. Indeed, the Haematozoa make up only a small part of numerous viral, bacterial, and fungal infectious diseases that threaten the health and well-being of organisms. How does one know that easily detectable blood parasites are the proper study organisms in the ecological context and that their health impact is not diminutive compared to the multitude of undiagnosed diseases?

An as yet untested possibility of approaching this question is to examine the health state of wild birds in relation to blood parasitemia. If blood parasites have a serious impact on the health of their hosts, then this ought to be revealed as a difference in health and immune parameters between infected and uninfected individuals. Alternatively, if other diseases are more important, so that their influence overrides that of blood parasites, then one should not expect differences in clinical parameters between infected and uninfected individuals. Accordingly, the aim of this study is to look for indications of the health impact of blood parasites on their avian hosts by examining the relationship between hemoparasite infection and various hemato-serological health state indices. The host species in this study was the great tit.

The great tit is a small (*c.* 19 g) short-lived hole-nesting passerine bird, among the most favoured research objects of avian ecologists, not least in the context of hemoparasite studies (Allander and Bennett 1994; Norris et al. 1994; Richner et al. 1995; Dufva 1996; Ots and Hõrak 1996; Oppliger et al. 1996, 1997; Allander 1997). Of the various genera of Haematozoa parasitising great tits, we concentrated on *Haemoproteus* sp. as the most common in our study area (Hõrak et al. 1998) and in wild birds generally (Atkinson and van Riper 1991). Importantly, the genus *Haemoproteus* has been widely used for testing the Hamilton-Zuk hypothesis, in particular by Hamilton and Zuk (1982) themselves. *Haemoproteus* belongs, together with *Plasmodium* and *Leucocytozoon*, to the group of intracellular sporozoan parasites that complete part of their complex life cycles within circulating red blood cells of vertebrate hosts. They are transmitted by blood-sucking Diptera, such as mosquitoes.

We predict that if *Haemoproteus* blood parasites do have a health impact on the host birds, parasitised individuals should differ from unparasitised ones with respect to their immune and general health parameters. If the immune system of breeding individuals is sensitive to *Haemoproteus*, we would expect the prevalence and intensity of infection to correlate positively with lymphocyte hemoconcentration (cell-mediated response) and plasma gamma-globulin concentration (humoral response). If non-specific, phagocytozing leukocytes are involved in fighting the infection in the bloodstream, we would expect a positive correlation between the parasite and heterophile concentration. If *Haemoproteus* damages its host by depletion of the erythrocyte pool, we would expect the infected individuals to develop anemia (low hematocrit values). Finally, if the infection is associated with malnutrition, liver disorders, or acute phase inflammatory responses, we would expect the parasitemia to correlate negatively with plasma albumin levels. In addition to hemato-serological parameters, we test for the association between hemoparasite infection and body mass, as the most commonly used non-specific index of individual condition. In this case, we have no directional predictions, because diseased individuals may either be leaner than healthy ones due to exhaustion of body reserves, or alternatively, heavier because they avoid losing mass in order to save body resources for anti-parasite immune response. Because a host's response to parasites may depend on various physiological, immunogenetic, or ecological factors, we will test whether the possible health impact of blood parasites differs between sex and age categories and between study sites in great tits breeding in two contrasting habitat types (urban and rural) situated close to one another.

Methods

The study was carried out during 1995–1997 in two neighbouring (urban and rural) great tit populations breeding in nestboxes in and near the city of Tartu, south-east Estonia (58°22′ N, 26°43′ E). The distance between the two study areas was 8 km. Study areas are described by Hõrak et al. (1995). Altogether, 513 individuals were used in the analyses.

Adult great tits were captured on their nests when nestlings were 8 days old. Birds were sexed and classified as yearlings or older (≥ 2 years) according to plumage characteristics as described by Svensson (1992). They were weighed with a Pesola spring balance with a precision of 0.1 g and their tarsi measured with sliding callipers to the nearest 0.1 mm. To reduce the influence of structural size and diurnal variation, body mass was expressed as the residual from a multiple linear regression of mass on cubed tarsus length and weighing time of day (measured from sunrise).

As complementary measures of health state, we employed simple clinical screening methods known to detect the disease process, although not providing any specific diagnostic tests. These clinical indices were:

1. Total and differential leukocyte counts. Elevated leukocyte count (leukocytosis) is characteristic to inflammatory processes in response to microbial and macroparasite infections (e.g., Dein 1986). Specifically, we concentrated on the two most numerous leukocyte types, namely heterophiles and lymphocytes.

Heterophiles are non-specific phagocytozing cells that enter the tissues during the inflammatory response. Lymphocytes elicit pathogen-specific immune response. T-lymphocytes (which make up the majority of circulating lymphocytes) play a key role in cell-mediated immunity, while B-lymphocytes that produce immunoglobulins are primarily responsible for antibody-mediated or humoral immunity.

2. Plasma protein concentrations. Decrease in total plasma protein concentration accompanies almost all diseases but is an especially prominent symptom of malnutrition and infections, especially when caused by a decline of plasma albumin level (e.g., Kawai 1973). A decrease of plasma albumin content during the inflammation is usually accompanied by a simultaneous increase of globulin (especially gamma-globulin) concentration. The gamma-globulin fraction of the plasma includes most of the known antibodies involved in immune response to protozoan, bacterial, and viral infections.

3. Hematocrit or packed cell volume (PCV). This index measures the relative amount of red blood cells in total blood volume. It is potentially interesting in the context of the present study because blood parasitism can result in low, anemic PCV values (Dein 1986).

All these measures were estimated from blood samples (100–150 µl) collected from the tarsal or brachial vein. For identification of blood parasites and leukocytes, a drop of blood was smeared on two individually marked microscope slides, air-dried, fixed in absolute methanol, and stained with azure-eosin. Slides were examined for presence and abundance of blood parasites under 400× magnification for *Trypanosoma*, microfilaria and *Leucocytozoon*, and under 1000× with oil immersion for *Haemoproteus* and *Plasmodium*. Because *Haemoproteus* sp. was the only common blood parasite, occurring in approximately half of the individuals (Hõrak et al. 1998), we confined our examination of the intensity of parasitemia to this genus.

After scanning for blood parasites, slides were used to estimate the total number and proportion of different types of leukocytes. The proportion of different types of leukocytes was assessed on the basis of an examination of a total of 100 leukocytes under oil immersion. Estimates of the total white blood cell count (WBC) and intensity of parasitemia were obtained by counting the number of leukocytes or parasites per approximately 10,000 erythrocytes. Differential leukocyte counts were obtained by multiplying their proportions with WBC. The repeatabilities of the leukocyte and parasite counts obtained from repeated scannings of the same blood smear were high (r = 0.96, 0.93, 0.87, and 0.86 for *Haemoproteus* intensity, heterophile count, WBC and lymphocyte count, respectively; all P values <0.00001; Ots et al. 1998). Moreover, measurement errors (estimated as variation coefficients obtained from the repeated sampling of the same individual) of all hemato-serological indices used in this study were reasonably low (between 0.06 and 0.25; Ots et al. 1998). Similar methods for estimating leukocyte concentrations have been also used by, e.g., Dufva and Allander (1995), Saino et al. (1995, 1997) and Saino and Møller (1996).

Hematocrit was measured with digital callipers to the nearest 0.1 mm after 10 min centrifugation of heparinized capillary tubes at 10,000 rpm. After determination of hematocrit, the plasma was separated from the blood cells and stored at −20°C until analyzed. 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 densitometrically scanned at 525 nm wave length. Total plasma protein concentration was determined in a photometric colorimetric test using the Biuret method. Because of difficulties in separating the prealbumin fraction from albumin, summed concentrations for both are reported and termed albumin concentration. Persons examining blood samples had no information about the phenotypic values of individuals.

Relationships between health state indices and parasitemia were examined in ANOVAs (comparison of infected and uninfected individuals) and ANCOVAs (intensity of parasitemia) using health parameters as dependent variables. To control for confounding factors such as sampling time, sex, age, and year of study, all models were checked for the significance of these terms (and their interactions with parasitemia). Additionally, we checked each model for the significance of the site × *Haemoproteus* interaction term, in order to test whether the hemoparasite effect on health state differed between study areas. Main effects were kept in models only when significant, except when testing the significance of interaction terms. Analyses were performed with the SAS GLM procedure (SAS Institute 1985), using type III sums of squares, enabling us to account for all effects of independent variables simultaneously. Log-transformed values of all parameters except hematocrit and residual body mass were used in order to normalise distribution of the dependent variables. Correspondence of residuals to normal distribution was checked with SAS UNIVARIATE procedure (Shapiro-Wilk's W-test); in no case was the assumption of normality violated. All significance levels refer to two-tailed tests. Subscripts used in connection with F-tests refer to degrees of freedom.

To avoid pseudoreplication, all analyses were performed in a test sample including birds that were captured only once and randomly picked observations of individuals that were captured in more than one study year. Thus, no bird was entered more than once in the analysis. Females incubating in empty nests were excluded from all analyses because they were known to differ from the "normal" birds with respect to their hematological parameters (Ots and Hõrak 1996).

Results

Great tits infected with *Haemoproteus* blood parasites had a significantly higher lymphocyte concentration in their peripheral blood than uninfected individuals (Table 1, Fig. 1). The parasite effect on lymphocytes evidently influenced also total leukocyte counts (WBC), because the heterophile concentration was not sensitive

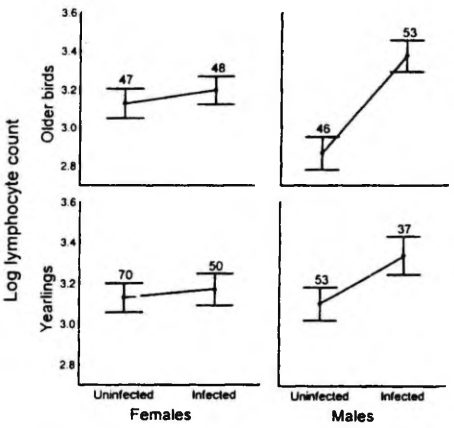

Fig. 1 Lymphocyte hemoconcentration of great tits in relation to infection status, sex, and age. Data are presented as least square means and standard errors calculated by the SAS GLM procedure, i.e., adjusted values, accounting for the confounding factors (from the ANOVA model: Lymphocyte count = *Haemoproteus*, Age, Sex, Time, Year, *Haemoproteus* × Age × Sex). In all figures, *numbers* at error bars denote sample sizes

Table 1 Effect of *Haemoproteus* prevalence on peripheral blood leukocyte counts of great tits. In all tables, *P*-levels greater than 0.1 are indicated as not significant (NS) and *Time* effect stands for two time categories (either birds captured at daytime while feeding nestlings, or roosting in the nestboxes). All dependent variables are log-transformed (*WBC* white blood cell count)

Effect	Lymphocyte count		Heterophile count		WBC	
	F_{df}	*P*	F_{df}	*P*	F_{df}	*P*
Haemoproteus	$19.6_{1,403}$	<0.001	$0.1_{1,403}$	NS	$9.3_{1,403}$	0.003
Sex	$0.2_{1,403}$	NS	$20.3_{1,403}$	<0.001	$7.4_{1,403}$	0.007
Sex × *Haemoproteus*	$11.1_{1,403}$	<0.001	$1.0_{1,403}$	NS	$6.6_{1,403}$	0.011
Year	$177.6_{1,403}$	<0.001	$95.8_{1,403}$	<0.001	$222.0_{2,403}$	<0.001
Time	$39.3_{1,403}$	<0.001	$6.5_{1,403}$	0.011	$31.6_{1,403}$	<0.001

Table 2 Effect of *Haemoproteus* intensity on peripheral blood leukocyte counts of great tits. Slope for the *Haemoproteus* effect on lymphocyte count = 0.05 ± 0.02 (SE). None of the interaction terms was significant at the 5% level. *Haemoproteus* stands for intensity of infection (only infected birds included). All dependent variables are log-transformed

Effect	Lymphocyte count		Heterophile count		WBC	
	F_{df}	*P*	F_{df}	*P*	F_{df}	*P*
Haemoproteus	$6.4_{1,184}$	0.012	$0.0_{1,183}$	NS	$3.2_{1,184}$	0.077
Year	$73.9_{2,184}$	<0.001	$38.4_{2,183}$	<0.001	$92.9_{2,184}$	<0.001
Time	$15.8_{1,184}$	<0.001	$3.8_{1,183}$	0.052	$20.7_{1,184}$	<0.001
Sex			$5.5_{1,183}$	0.020		

Table 3 Effect of *Haemoproteus* prevalence on plasma proteins of great tits. None of the interaction terms was significant at the 5% level. All dependent variables are log-transformed

Effect	Total protein		Albumin		Gamma globulin	
	F_{df}	*P*	F_{df}	*P*	F_{df}	*P*
Haemoproteus	$6.2_{1,308}$	0.029	$0.0_{1,268}$	NS	$10.7_{1,263}$	0.001
Age					$10.5_{1,263}$	0.001

to infection. The significant *Haemoproteus* × sex interaction term in Table 1 indicates that the parasite effect was sex-specific. This was because infection status did not affect the peripheral lymphocyte count of females ($F_{1,211} = 0.58$, $P = 0.4$ when analyzed separately). Figure 1 shows that the effect of *Haemoproteus* was also age-specific: among old males the difference in lymphocyte counts between infected and non-infected individuals was more prominent than among yearlings. The simultaneous age- and sex-specific effect of infection status was also statistically significant when the *Haemoproteus* × sex interaction term in the model was replaced by the *Haemoproteus* × sex × age interaction term ($F_{4,393} = 4.22$, $P = 0.002$).

Intensity of *Haemoproteus* infection correlated positively with lymphocyte hemoconcentration (Table 2). However, unlike for parasite prevalence, no sex- and age-specific effects could be detected ($P = 0.8$ for both the sex × *Haemoproteus* and age × *Haemoproteus* interaction terms). As in the case of prevalence, the parasite effect on lymphocytes resulted in a (marginally significant) positive correlation between parasite and total leukocyte counts, while heterophile concentration did not correlate with intensity of infection (Table 2).

Infected birds had higher total plasma protein concentrations than uninfected birds. This was entirely due to the parasite effect on the plasma globulin fraction, as the albumin level was not affected by infection status (Table 3). Infected individuals had higher plasma gamma-globulin content within both age categories (Fig. 2). We failed to find any evidence that infection status had affected plasma proteins in sexes or age classes differently because none of corresponding interaction terms in the models was significant at the 5% level. Intensity of *Haemoproteus* did not correlate significantly with any of the protein variables examined (all *P* values >0.2; n = 116–130).

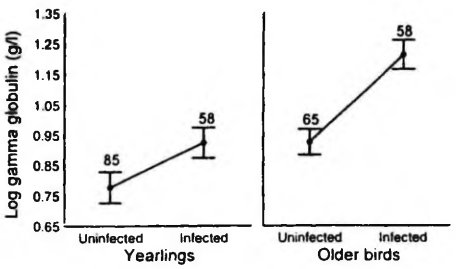

Fig. 2 Plasma gamma-globulin concentration (mean ± SE) of great tits in relation to infection status and age

Table 4 Effect of *Haemoproteus* prevalence on residual body mass of great tits. *Site* stands for two study areas located in different (urban and rural) habitats

Effect	F_{df}	P
Haemoproteus	$2.2_{1,501}$	NS
Year	$7.8_{2,501}$	< 0.001
Site	$1.0_{1,501}$	NS
Site × Year × *Haemoproteus*	$2.2_{7,501}$	0.030

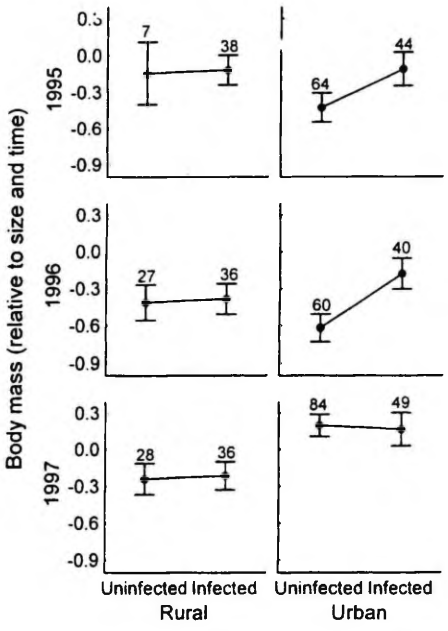

Fig. 3 Residual body mass (mean ± SE; adjusted for body size and weighing time) of great tits in relation to infection status, year and study site (in rural or urban habitat)

Hematocrit was not associated with infection status ($F_{1,302} = 0.06$, $P = 0.9$) in a model adjusting for site, year and sex differences. Neither did it correlate with intensity of parasitemia ($F_{1,127} = 0.05$, $P = 0.8$) in an analogous model.

Infection status affected body mass of great tits in a year- and site-dependent manner (Table 4). Figure 3 shows that infected birds in the urban study area were heavier than uninfected ones in 1995 and 1996 but not in 1997. In none of the models was body mass related to intensity of parasitemia (always $P > 0.9$).

Discussion

Our results clearly demonstrate that great tits with the gametocytes of *Haemoproteus* in their peripheral blood differed from unparasitised ones with respect to several clinical parameters. Does this mean that the blood profiles of these birds were directly affected by *Haemoproteus*, or was the parasitemia a by-product of a generally poor heaith state of certain individuals? The latter alternative seems unlikely because it would mean that parasite-free birds had suppressed the relapse of infection. If this were the case, we would have detected elevated immunoglobulin levels (known to remain high for weeks post infection) in their blood. On the contrary, uninfected individuals had low gamma globulin levels (Fig. 2), indicating that successful suppression of the spring relapse (that coincides with breeding) of infection was not the cause of their parasite-free status. We therefore conclude that *Haemoproteus* blood parasites affected the health parameters examined of their great tit hosts. To our knowledge, this is the first evidence of a health impact of hemoparasite infections in a wild bird species breeding under natural conditions. Previous reports on the pathogenicity of avian hemoparasites have been based on anecdotal evidence of high parasite loads of moribund individuals, or on (mostly laboratory) studies concerning birds in farms or zoological gardens (see Bennett and Bishop 1988; Atkinson and van Riper 1991; Bennett et al. 1993 for reviews). Such data cannot be used to assess the ecological importance of these parasites. As shown in this study, avian blood parasites have a severe impact on their wild hosts, because the relationships between *Haemoproteus* infection and immune and condition indices would not have been detected unless the parasite effect on the hosts' health state is really important in comparison with other diseases.

What are the physiological and ecological implications of the findings of the present study? Although the non-specific design of the hematological assay procedures we have used prevents detailed inferences to be made regarding the mechanisms of the hosts' response to *Haemoproteus* infection, some tentative conclusions concerning the nature of parasite impact on the physiology of breeding great tits can be drawn.

First, the results that infected individuals revealed both elevated lymphocyte hemoconcentration and gamma-globulin levels indicate that both cell-mediated and humoral response mechanisms are involved. Notably, the intensity of parasitemia correlated with lymphocyte hemoconcentration but not with gamma-globulin level. This result is consistent with those of Graczyk et al. (1994a, b), who failed to find a correlation between parasite intensity and antibody titre against anti-*Haemoproteus* and anti-*Plasmodium* immunoglobulins in pigeons and penguins. This can be explained in terms of the diversity of immune mechanisms against different stages of parasite: antibodies play a significant role in combat against exo-erythrocytic stages (see Graczyk et al. 1994a; Wakelin 1997), while the relapse of infection in the bloodstream is controlled by cell-mediated immunity (Graczyk et al. 1994a). In this case, the fact that the intensity of parasites in the bloodstream not necessarily reflects the exo-erythrocytic infection

(Atkinson and van Riper 1991) might well explain the lack of correlation between parasite intensity and gamma-globulin level found in the present study.

Another study of breeding great tits (Dufva and Allander 1995) found no evidence for any association between parasites and leukocytes. This was possibly due to a much lower sample size (45 individuals) than in the present study.

Second, the heterophile concentration in the peripheral blood was not associated with parasite prevalence or intensity. Peripheral heterophilia is a symptom recurring in almost any kind of stress (e.g., Maxwell 1993), inflammation (Parslow 1994), and infection, especially microbial challenge (Rose et al. 1979; Hawkey et al. 1985). Our data therefore suggest that *Haemoproteus* infection (specifically, its blood stage) was not associated with an increased stress level and acute phase response of inflammation.

Third, plasma albumin levels were not related to *Haemoproteus* infection. Low albumin levels are indicative of disruption of protein metabolism, either due to starvation, or protein malnutrition (Coles 1980), or decreased synthesis of albumin in the liver, which is a specific symptom of acute phase response of inflammation (e.g., Fleck 1989). Thus, the results of plasma albumin analysis support our conclusion that *Haemoproteus* infection does not cause severe inflammatory response. Additionally, they indicate that infected individuals do not reveal symptoms of poor nutritional state.

Fourth, the lack of a relationship between hematocrit values and infection indicates that *Haemoproteus* blood parasites do not cause anemia in their hosts by depleting the erythrocyte pool. Similar results were obtained by Atkinson et al. (1988) and Dufva and Allander (1995) for different *Haemoproteus* species, while other haemosporidians (*Plasmodium* and *Leucocytozoon*) have been reported to cause anemia in their avian hosts by destruction of red blood cells (see Atkinson and van Riper 1991 for a review).

Sex differences

We could not detect any sex-related effects of parasite infection on the gamma-globulin level, while the lymphocyte hemoconcentration was affected by infection status only in males but not in females (Fig. 1). Possibly, this might be related to the differential role of sex hormones in the immune response of the host. It is generally accepted that testosterone suppresses immunity and increases host susceptibility to many parasites, particularly by affecting T-cell functions (e.g., Ahmed et al. 1985; Yamamoto et al. 1991; Wunderlich et al. 1992; Leitner et al. 1996). One might therefore speculate that the response of males to infection (in terms of increased lymphocyte hemoconcentration) might be stronger than that of females because males have to make a greater lymphopoietic effort than females in order to fight the relapsing infection.

Age differences

The lymphocytic parasite response in male great tits differed between yearlings and older birds. Figure 1 shows that the difference was caused by particularly low lymphocyte counts of uninfected old individuals. One possible explanation might be that yearlings mount a generally stronger cell-mediated immune response (to other diseases) than older birds, either because young age classes are more susceptible to infections for immunogenetic reasons (selective mortality), or because older birds have acquired immunity during their longer exposure to pathogens. Alternatively, an ecological explanation would be that uninfected old individuals have low lymphocyte counts because they "prefer" to allocate more resources to reproduction (terminal reproductive effort) instead of safeguarding against possible immune challenges by keeping the number of patrolling T-lymphocytes in the bloodstream high. Unfortunately, we cannot discriminate between these possible explanations because we do not know whether the high peripheral lymphocyte count reflects only the current level of immune response or also the potential for immune defence in case of occasional pathogenic challenge.

Although both age classes responded to *Haemoproteus* infection with gammaglobulinemia, yearlings had generally lower gamma-globulin level than older birds (Fig. 2). Probably, this indicates that older individuals are able to mount a more efficient humoral immune response than yearlings.

Site differences

In 2 of 3 study years, the *Haemoproteus* infection was associated with higher body weights of the birds in the urban, versus rural study area (Fig. 3). Because the study sites are not isolated by distance, the difference in responsiveness of urban and rural birds can hardly be ascribed to genetic factors. Thus, the reasons why infection was differently associated with body mass of urban and rural great tits are likely to be ecological. The tendency of infected individuals being heavier than non-infected ones suggests that they avoided losing body mass in order to save resources for anti-parasite immune response. This result would indirectly support the hypothesis that birds may benefit from reducing their body mass during brood rearing because it will enable them to reduce their flight costs and hence, to save energy for self maintenance and food provisioning (Freed 1981; Norberg 1981). If the mass change represents an optimal balance between the costs and benefits of low body mass (Nur 1984; Hillström 1995), then one might expect that healthy individuals can afford to reduce their body mass to a greater extent than diseased ones. Why, then, was the body mass of rural great tits not related to their infection status? One possible explanation is that great tits in our rural study area pursue a reproductive tactic with a relatively greater propensity of investing in the

current reproductive attempt as compared to their urban conspecifics. Our rural great tit population is characterised by higher reproductive rate (Hõrak 1993; Hõrak et al. 1995) but lower adult survival (Hõrak and Lebreton 1998) as compared to the neighbouring urban great tit population in Tartu. Moreover, the intense reproductive effort of rural birds seems to impose interannual reproductive costs on the parents, because females who rear all or most of their nestlings to fledging survive worse than females with high nestling mortality (Hõrak 1995). Compared to urban birds, rural great tits had also significantly higher hematocrits (Ots et al. 1998) possibly indicative of exercise-induced polycythemia due to a high work load (Hõrak et al. 1998). It is therefore possible that infected rural great tits, unlike their urban conspecifics, did not invest their resources to parasite defence because they were generally more inclined towards making a terminal reproductive effort.

We are aware of only one earlier study reporting a significant (negative) association between hemoparasite infection and parental body mass in wild birds (Bosch et al. 1997; intensity of *Haemoproteus lari* infection in breeding female yellow-legged gulls).

Conclusions

Our results on the health impact of *Haemoproteus* on breeding great tits present strong evidence for a potentially important ecological impact of blood parasites on hosts in natural bird populations. This indicates that avian blood parasites offer a model system that is highly suitable for testing hypotheses regarding sexual selection and physiological mechanisms of reproductive costs. However, our results also show that the impact of parasites on various general health and immune parameters can vary with sex, age, and possible ecological factors, and this calls for careful considerations of confounding variables in avian hemoparasite studies.

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Haematological health state indices of reproducing Great Tits: a response to brood size manipulation

P. HÕRAK,* I. OTS‡ and A. MURUMÄGI‡

**Estonian Institute of Zoology and Botany, Riia 181, EE2400 Tartu, Estonia and Department of Zoology, Uppsala University, Villavägen 9, S-75236 Uppsala, Sweden, and ‡Department of Zoology and Hydrobiology, Tartu University, Riia 181, EE2400 Tartu, Estonia*

Summary

1. A brood size manipulation experiment was performed in two Great Tit (*Parus major* L.) populations in order to evaluate the effect of raising different numbers of nestlings on parental health state.

2. Brood enlargement resulted in elevated heterophile:lymphocyte ratios and decreased lymphocyte number in the peripheral blood, indicating that increased reproductive effort causes immunosuppression.

3. Haematocrit increased in response to brood enlargement, suggesting a response to the requirement of elevated oxygen-carrying capacity of the blood during increased work load.

4. Parental body mass revealed a tendency to decrease in response to brood enlargement.

5. No effect of brood size manipulation on total leucocyte count, heterophile count, intensity of *Haemoproteus* blood parasite infection or plasma proteins could be detected.

6. Health state indices were more sensitive to brood size manipulation in the Great Tits breeding in a rural habitat than urban birds.

Key-words: Body mass, haematocrit, leucocytes, *Parus major*, reproductive effort

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Introduction

Many life-history studies have been devoted to finding explanations for patterns in reproductive effort (RE). Ironically, however, the question of how to measure or even how to define reproductive effort is still open to much debate (Hirshfield & Tinkle 1975; Stearns 1992). The traditional approach to measuring RE that most easily can be applied to altricial birds is manipulation of brood size and subsequent measurement of parental reactions in terms of provisioning rate, body mass or energy expenditure. The question to what extent (if at all) the increased reproductive effort actually confers deleterious effects upon parents, however, often remains unknown in such studies (e.g. Verhulst 1995; Orell *et al.* 1996). To solve this problem, an assessment of the direct effects of brood size manipulations on parental health state is therefore necessary. Existing reports on brood manipulation effects on parental health state include measurements of stress hormone levels (Silverin 1985; Hegner & Wingfield 1987), blood parasitaemia (Norris, Anwar & Read 1994; Allander 1995; Richner, Christe & Oppliger 1995; Ots & Hõrak 1996) and leucocyte profiles (Ots & Hõrak 1996). However, none of these has reported several health indices simultaneously, and therefore to determine which state indices are most sensitive requires further characterization.

The aim of this study is to describe the reaction of 11, mostly haemato-serological, parental health state indices to brood size manipulations in two Great Tit populations breeding in different habitats. Traits examined include plasma protein concentrations, estimates for total and differential leucocyte counts, blood parasitaemia, haematocrit and parental body mass. This paper is a companion to another one (Ots, Murumägi & Hõrak 1998), in which we describe diagnostic meaning, measurement precision and sources of natural variation of the same parameters.

Methods

The fieldwork was carried on during the breeding period of 1996 in two neighbouring (urban and rural) Great Tit populations breeding in nestboxes in and near the city of Tartu, south-east Estonia (58°22′N, 26°43′E). The study area is described in Hõrak *et al.* (1995).

MEASUREMENT OF HEALTH STATE INDICES

Adult Great Tits were captured in their nests when their nestlings were 8 days old. They were weighed with a Pesola spring balance with a precision of 0·1 g and their tarsi measured with a sliding calliper to the nearest 0·1 mm always by the same person (PH). In an attempt to separate mass from structural size and to take into account diurnal mass changes, residual masses were computed as the residuals from multiple linear regressions of mass on cubed tarsus length and weighing time (measured from sunrise).

Blood samples (100–150 µl) for haematological measurements were taken from the tarsal or brachial vein. For identification of blood parasites and leucocytes, a drop of blood was smeared on two individually marked microscope slides, air-dried, fixed in absolute methanol and stained with azure-eosin. Slides were examined for the presence and abundance of blood parasites under 400x magnification for *Trypanosoma* and *Leucocytozoon*, and under 1000x with oil immersion for *Haemoproteus* and *Plasmodium*. Because *Haemoproteus* sp. was the only common blood parasite, occurring in 47% (88/188) of the individuals, our examination of intensity of parasitaemia was confined to this genus.

After scanning for blood parasites, slides were also used to estimate the total number and the proportion of different types of leucocytes. The proportion of different types of leucocytes was assessed on the basis of an examination of a total of 100 leucocytes under oil immersion. Estimates for the total white blood cell count (WBC) and intensity of parasitaemia were obtained by counting the number of leucocytes or parasites per ≈ 10 000 erythrocytes. Differential leucocyte counts were obtained by multiplying their proportions with WBC. In the analyses, only data for lymphocytes and heterophils are used.

As an additional estimate of WBC the relative amount of leucocytes in total blood volume was used, measured as the height of the 'buffy coat' layer in a haematocrit capillary tube (Wardlaw & Levine 1983). Buffy coat layer and haematocrit (the relative amount of red blood cells in total blood volume) were measured with a digital calliper to the nearest 0·05 mm after 10 min of centrifugation of heparinized capillary tubes at 10 000 rpm.

Plasma samples for protein electrophoresis were obtained by centrifuging blood for 10 min at 3000 rpm, after which the plasma was stored at – 20 °C until analysed. 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 densitometrically scanned at 525 nm wavelength. Total plasma protein concentration was determined in a photometric calorimetric test using the Biuret method. Because of difficulties of separating the prealbumin fraction from albumin, summed concentrations for both are reported and termed albumin concentration.

The persons examining blood samples (IO for Haematozoa, haematocrit, buffy coat and plasma, and AM for leucocytes) had no information about the individual birds except ring number.

BROOD SIZE MANIPULATION

For the brood size manipulations, two 2-day-old (day 0 = day of hatching) nestlings were moved from 'reduced' nests to 'enlarged' nests with similar hatching date and clutch size, while a third nest with similar parameters was considered a control within the study site. Additional reduced and control clutches were created by moving one nestling from broods where one egg did not hatch to control broods where one egg did not hatch in order to maximize differences among manipulation categories. All experimental broods produced in this way were within the size range found naturally. Experimental and control broods did not differ significantly in respect of clutch size or hatching date (P diff. = 0·3...1).

Only broods where all eggs hatched and all nestlings were alive on the second day were chosen for enlargement. While this design can be interpreted as giving enlarged broods to a non-random sample of individuals, the alternative possibility of also increasing broods with unhatched eggs and early nestling mortality would have reduced our ability to detect the effects of brood enlargement. This is because differences in the number of nestlings between enlarged and control broods would have been reduced in this case. We argue that our results in respect of brood enlargement are conservative because increasing brood size is expected to have adverse effects on parents. Therefore, the inclusion of individuals being in some way 'superior' to others in the 'increased' category would deflate rather than inflate the experimental effects. Moreover, repeating the analysis excluding all birds with unhatched eggs resulted in all significance levels being similar to these reported in Table 1, suggesting that non-random allocation of birds among treatments did not affect the results.

Another possible source of sampling bias was avoided by exclusion of all clutches where more than one egg did not hatch from the analysis of experimental data. Alternatively, these birds could have been included in the 'reduced' manipulation category, but this might have led to a greater proportion of 'inferior' individuals among 'reduced' broods, which potentially could deflate the 'positive' effects of decreasing brood size. Additionally, it was checked that inclusion of birds with unhatched eggs in the 'reduced' category in the analyses would not have altered the conclusions.

Statistical analyses were performed using the SAS statistical package (SAS Institute 1985). Effects of manipulation were tested in ANOVAS (Proc GLM), using III type of sums of squares, enabling us to

Table 1. Effect of brood size manipulation on health state indices of the Great Tit. None of the interaction terms was significant at the 5% level. *P*-levels greater than 0·1 are indicated as not significant (NS). Time effect stands for two time categories (either birds captured at daytime while feeding nestlings or roosting in the nestboxes) and is included in models only when significant. *Haemoproteus* stands for intensity of *Haemoproteus* infection (only infected birds included). All trait values except haematocrit and residual mass are log-transformed. Traits are presented in decreasing order of the significance of the manipulation effect

	Lymphocyte (L) count		Haematocrit (H)		H:L ratio		Body mass	
Effect	F_{DF}	*P*	F_{DF}	*P*	F_{DF}	*P*	F_{DF}	*P*
Manipulation	$5{\cdot}0_{2,138}$	0·008	$4{\cdot}8_{2,113}$	0·010	$3{\cdot}5_{2,141}$	0·030	$2{\cdot}6_{2,139}$	0·081
Site	$0{\cdot}8_{1,138}$	NS	$33{\cdot}3_{1,113}$	< 0·001	$0{\cdot}3_{1,141}$	NS	$0{\cdot}1_{1,139}$	NS
Sex	$1{\cdot}6_{1,138}$	NS	$13{\cdot}0_{1,113}$	0·001	$11{\cdot}1_{1,141}$	0·002	$0{\cdot}1_{1,139}$	NS
Time	$18{\cdot}2_{1,138}$	< 0·001						
	WBC		Buffy coat		Heterophile count		*Haemoproteus*	
Effect	F_{DF}	*P*	F_{DF}	*P*	F_{DF}	*P*	F_{DF}	*P*
Manipulation	$1{\cdot}3_{2,138}$	NS	$0{\cdot}6_{2,104}$	NS	$0{\cdot}4_{2,138}$	NS	$0{\cdot}0_{2,62}$	NS
Site	$1{\cdot}1_{1,138}$	NS	$2{\cdot}4_{1,104}$	NS	$1{\cdot}2_{1,138}$	NS	$2{\cdot}1_{1,62}$	NS
Sex	$0{\cdot}3_{1,138}$	NS	$0{\cdot}3_{1,104}$	NS	$5{\cdot}4_{1,138}$	0·021	$7{\cdot}0_{1,62}$	0·010
Time	$16{\cdot}5_{1,138}$	< 0·001	$3{\cdot}7_{1,104}$	0·056	$6{\cdot}8_{1,138}$	0·010		
	Total protein		Albumin		Alb/Glo ratio			
Effect	F_{DF}	*P*	F_{DF}	*P*	F_{DF}	*P*		
Manipulation	$0{\cdot}8_{2,118}$	NS	$0{\cdot}1_{2,84}$	NS	$0{\cdot}2_{2,84}$	NS		
Site	$13{\cdot}4_{1,118}$	< 0·001	$7{\cdot}7_{1,84}$	0·007	$0{\cdot}4_{1,84}$	NS		
Sex	$0{\cdot}1_{1,118}$	NS	$2{\cdot}2_{1,84}$	NS	$5{\cdot}8_{1,84}$	0·019		

account for all effects of independent variables simultaneously. Log-transformed values of all parameters except haematocrit and residual body mass were used in order to obtain normal distribution of data values. Untransformed values were used for haematocrit and residual mass because these values were normally distributed. All significance levels refer to two-tailed tests. Only data from genuine first clutches were used.

Results

Three of 11 traits were significantly affected by the brood manipulation experiment, while one trait (body mass) revealed a tendency (albeit not significant below 5% level in the two-tailed test) of being affected (Table 1).

Brood enlargement resulted in a remarkable decrease in lymphocyte number in peripheral blood (lymphopenia) among both females and males with enlarged broods in the rural population, while no consistent trend could be detected in the urban population (Fig. 1). The heterophile:lymphocyte ratio was highest among rural females with enlarged broods (Fig. 2). In the rural population, the haematocrit of both females and males was higher in enlarged as compared with control and reduced broods. As was the case for the lymphocyte count, no clear pattern emerged in the city (Fig. 3). Body mass showed a tendency of decrease with increasing manipulation score in all cases except for urban males (Fig. 4).

No evidence for a brood manipulation effect on total leucocyte count, heterophile count, buffy coat layer, intensity of *Haemoproteus* blood parasite infection and plasma proteins was detected.

Discussion

Two immunological parameters, namely lymphocyte count and heterophile:lymphocyte ratio, were significantly affected by our brood size manipulation experiments. Lymphocytes are immune cells that assist in the recognition and destruction of many types of pathogens. Lymphocyte number in the peripheral blood is known to decrease in response to different stressors (Dein 1986; Dohms & Metz 1991), and particularly after strenuous physical activities (e.g. Hoffman-Goetz & Pedersen 1994), either because of glycocorticoidal reduction of lymphoid tissues (e.g. Dohms & Metz 1991) or because of emigration of lymphocytes from peripheral blood to other compartments of the organism (Dhabhar *et al.* 1995). Decreased lymphocyte number signals immunosuppression, which, to the detriment of the host, may enable microorganisms, particularly viruses, to evade early immunological reaction and establish infection. Lymphopenia in response to brood enlargement in our study therefore can be taken as evidence that increased reproductive effort may lead to potentially hazardous immunosuppression. It should be noted, however, that although several authors have suggested

that lymphopenia leads to increased mortality because of decreased resistance to viral pathogens, the experimental evidence for this mechanism has not yet been presented (e.g. Dabbert, Lochmiller & Teeter 1997).

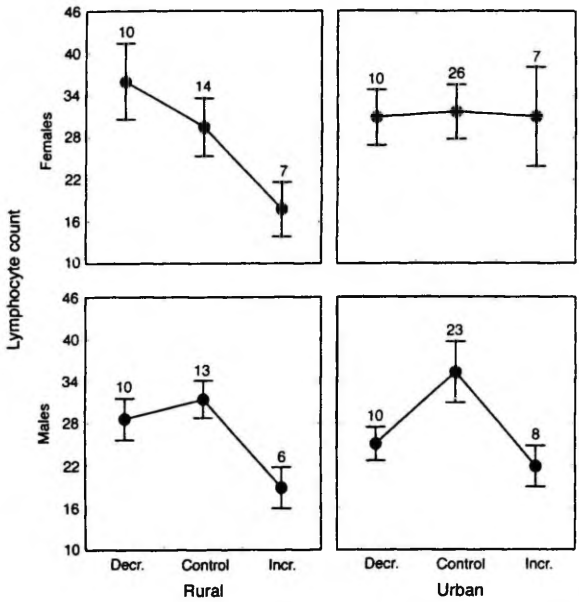

Fig. 1. Effect of brood size manipulation on parental lymphocyte count in two Great Tit populations. Decr. stands for decreased broods and incr. for increased broods. Untransformed data, presented as mean ± SE. Numbers indicate sample sizes.

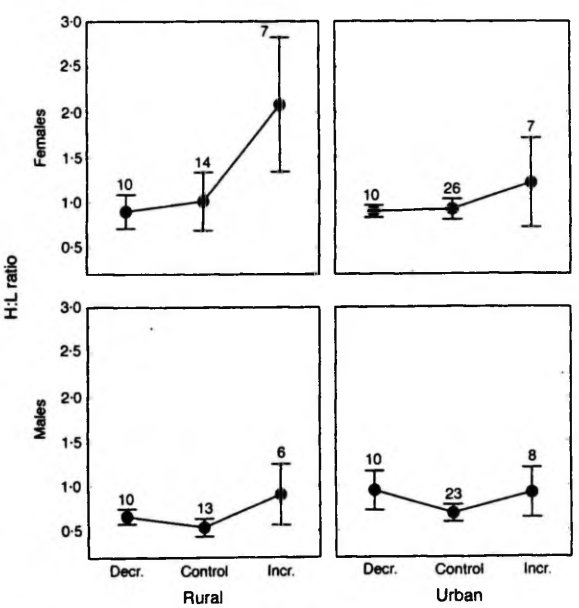

Fig. 2. Effect of brood size manipulation on parental heterophile:lymphocyte ratio in two Great Tit populations. Legend as in Fig. 1.

Heterophile:lymphocyte ratio is widely used as a stress index in poultry (Gross & Siegel 1983; Maxwell 1993), and is known to increase in response to various stressors, as a result of a decrease in the lymphocyte number on one hand and a simultaneous increase in the heterophile number on the other. Because the function of heterophils is phagocytosis of bacteria, an elevated number of heterophils may be interpreted as enhancing host resistance to bacterial infections. However, a negative side-effect will be tissue damage due to inflammatory processes (e.g. Fulton *et al.* 1996; Suzuki *et al.* 1996). Our finding of an increased heterophile:lymphocyte ratio in response to brood enlargement probably supports the applicability of heterophile:lymphocyte ratio as an indicator of the stressfulness of the reproductive effort of birds under natural conditions (see also Ots & Hõrak 1996).

Leucocyte responses to brood manipulation observed in our study were similar to those induced by injection of stress hormone (corticosterone) in poultry. In this context, our results are similar to those of Silverin (1985), who found elevated corticosterone levels in Pied Flycatchers (*Ficedula hypoleuca*) in response to brood enlargement. A similar tendency was observed in female House Sparrows (*Passer domesticus*) by Hegner & Wingfield (1987).

The third haematological parameter affected by brood manipulation was haematocrit, which increased in response to brood enlargement. It is suggested that this reflects an exercise-induced polycythaemia (e.g. Hsia *et al.* 1995; Toll *et al.* 1995; Piersma, Everaarts & Jukema 1996), as a response to the requirement of an elevated oxygen-carrying capacity of the blood during increased work load. This idea is supported by the finding that haematocrits were most elevated among the increased broods of rural Great Tits (Fig. 4) which, contrary to urban birds, also fledged more nestlings than controls (P. Hõrak & I. Ots, unpublished observations), and thus were likely to work most intensively. Notably, an association between elevated haematocrit and increased work load was also found in a study of Barn Swallows (*Hirundo rustica*) by Saino *et al.* (1997). These findings offer an alternative explanation to the results presented by Dufva (1996), who interpreted the positive correlation between haematocrit and clutch size in the Great Tit as indicative of poor health state of females laying small clutches. We would rather suggest that a positive correlation occurred because females with larger clutches had higher haematocrits because of their increased oxygen consumption associated with the higher work load incurred while feeding larger broods.

In addition to haematological parameters, brood manipulation also affected parental body mass. Although the experimental effect was not significant in a two-tailed test, the difference went in the expected direction, suggesting mass loss accompanying the increased work load.

Plasma proteins and estimates of total leucocyte count, heterophile number and intensity of *Haemoproteus* infection were not demonstrably affected by the brood manipulations. The observation that brood manipulation did not affect parasitaemia differs from the results of previous Great Tit studies by Norris *et al.* (1994), Allander (1995) and Richner *et al.* (1995). Moreover, it is inconsistent with our own results from the previous year (Ots & Hõrak 1996), when experimental reduction of brood size resulted in decreased *Haemoproteus* parasitaemia among rural female Great Tits. One possible explanation would be that 1996 was such an extremely favourable year for breeding that the rearing of unmanipulated broods might have demanded less effort than in the previous year. However, comparison of parasite infections among years does not provide strong support for this assumption because the prevalence of *Haemoproteus* parasitaemia was only slightly higher in 1995 (54%, 110 of 203 individuals; Ots & Hõrak 1996) than in 1996 (47%, 88 of 188 individuals) and also the intensities of parasitaemia among infested individuals did not differ significantly among years (I. Ots & P. Hõrak, unpublished observations).

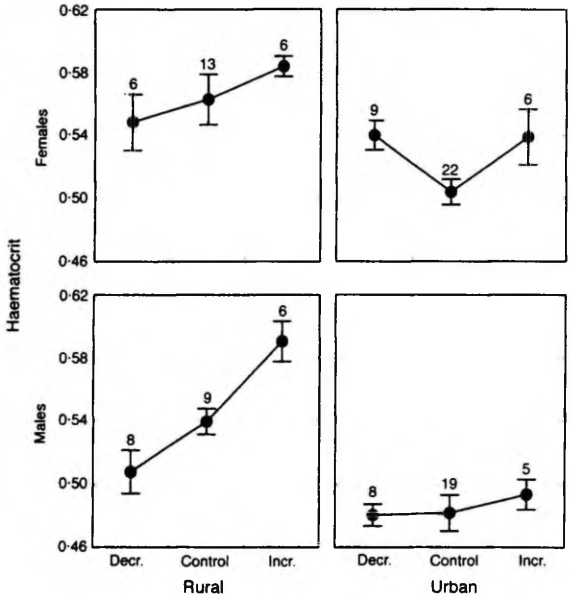

Fig. 3. Effect of brood size manipulation on parental haematocrit in two Great Tit populations. Legend as in Fig. 1.

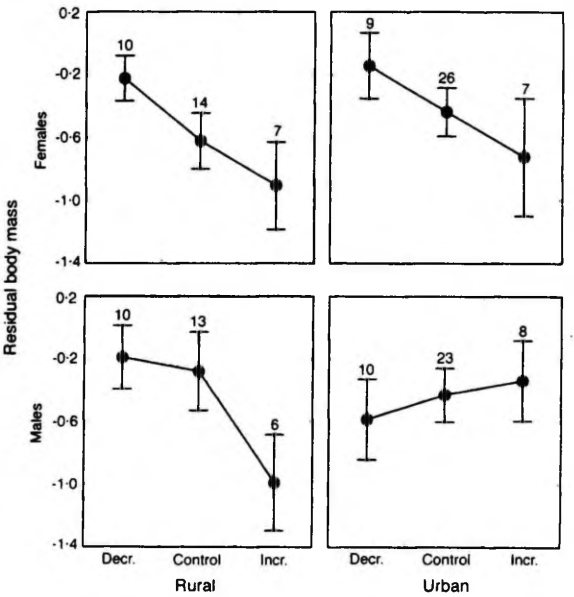

Fig. 4. Effect of brood size manipulation on parental body mass (residual in respect to time and size) in two Great Tit populations. Legend as in Fig. 1.

Common to all four parameters significantly affected by the brood manipulation was that manipulation effects were clear in the rural population but not in the city (Figs 1–4). This finding agrees with our previous results, according to which a relationship between reproductive effort and *Haemoproteus* parasitaemia could be demonstrated in the rural Great Tits but not in city population (Ots & Hõrak 1996). These results are surprising especially in view of the fact that the clutch size of the rural Great Tits on average was two eggs larger than in the urban population, which means that adding or removing two nestlings was a proportionally larger modification of the parental effort in the city than in the countryside.

One possible explanation of the greater responsiveness of rural rather than urban Great Tits might be that the effect of the brood manipulation on parental haematological parameters was masked by a generally worse health state of urban birds. Urban Great Tits are probably more seriously food-limited during breeding because they lay on average two fewer eggs and fledge three fewer nestlings than their rural conspecifics (Hõrak 1993a). The seasonal decline in egg size (Hõrak *et al.* 1995) and clutch size (Hõrak 1993b) in urban, but not rural, Great Tits in our study areas also suggests that urban birds in Tartu are subjected to more severe proximate reproductive constraints. On the other hand, the hypothesis of generally worse health state of urban birds is contradicted by our observation of their higher average blood albumin level, which is a symptom of good health (Ots *et al.* 1998). We would therefore like to propose an alternative explanation, namely that the Great Tits in our rural study area pursue a reproductive tactic with a relatively greater propensity of investing in the current reproductive attempt than the urban Great Tits in Tartu. This explanation is supported by our finding of significantly higher haematocrits of our rural Great Tits, possibly indicative of exercise-induced polycythaemia due to a very high work load. Notably, the

females raising a greater number of young were less likely to survive in our rural Great Tit population but not in our urban one (Hõrak 1995), in which the adult survival was also generally higher (Hõrak & Lebreton 1998).

What is the relevance of this study for the detection of the physiological impact of reproductive effort?

First, our result that brood manipulation markedly affected the haematological traits only in one of the two Great Tit populations indicates that the relationships between rearing broods of manipulated size and parental physiology are by no means ubiquitous. This result implies the necessity of examination of the physiological effects of reproductive effort in populations breeding under different environmental conditions. Even so, the responsiveness of individuals to brood size manipulation may differ among years within the same population, as indicated by the presence of experimental effect on *Haemoproteus* parasitaemia in 1995 but lack of such an effect in 1996 in our rural study area. Notably, there are also some inconsistencies among the results of other studies demonstrating the effect of brood size manipulations on haematozoan infection. Only one study (Allander 1995) found that brood manipulation had similar effects on *Haemoproteus* infection on both male and female Great Tits. Richner *et al.* (1995) and Norris *et al.* (1994) showed that brood enlargements resulted in increased parasitization on male but not female Great Tits, while Ots & Hõrak (1996) found the experimental effect only among females. There are several factors that might obscure the relationship between reproductive effort and parasitization, especially in the case of small sample sizes. Brood size manipulations are expected to result in increased parasitization if (1) individuals change their reproductive effort in response to manipulation, (2) premanipulative health state and predisposition to infections of all individuals is similar and (3) reproductive effort affects the immune response of all individuals in the same manner. The second and third assumptions do not hold in the presence of individuals that do not develop infection simply because they have not been occasionally infested by vectors of blood parasites (blood-sucking Diptera). Additionally, differential response to brood size manipulation might occur if some individuals possess acquired or inherited immunity to parasites. In that respect, other haematological state indices appear more suitable for the rough estimation of individuals' health state than parasitization. This conclusion is supported by the presence of experimental effect on heterophile:lymphocyte ratios in our study in both 1995 (Ots & Hõrak 1996) and 1996.

Second, although it is relatively well established by now that increased reproductive effort changes the physiological condition of individuals, it does not necessarily mean that these changes imply any ecological costs in terms of reduced future survival or fecundity. Indeed, the evidence for the effect of reproductive effort on health state predominantly originates from the parasitological studies (reviewed in Møller 1997), and the harmfulness of blood parasite infections in wild birds has been questioned (Bennett, Peirce & Ashford 1993). Moreover, there has been no indication that rearing of enlarged broods results in reduced adult survival in Great Tits (Pettifor, Perrins & McCleery 1988) and for other species such an evidence is scarce as well (e.g. Lessells 1991; Stearns 1992).

Forthcoming studies will probably elucidate the problem whether the non-parasitic hemato-serological health state indices can be used for prediction of an individual's survival and future reproductive performance. Another promising perspective for examining the physiological costs of reproduction would be measuring immunocompetence by challenging individuals with novel antigens such as sheep red blood cells (SRBC). A few first studies in this area have revealed exciting results. Deerenberg (1996) demonstrated that captive Zebra Finches (*Taeniopygia guttata*) raising enlarged broods were less able to raise immune response against SRBC while Saino, Bolzern & Møller (1997) showed that male Barn Swallows that showed the highest immune response to SRBC were more likely to survive until the breeding season following that in which they have been inoculated. In addition to the possibility of examining the relationships between immunocompetence and survival, such studies will also enable the testing whether individuals' ability to produce immune response can be predicted on the basis of simple haematological indices.

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Great tits *Parus major* trade health for reproduction

INDREK OTS[1] AND PEETER HÕRAK[1,2]*

[1] *Animal Ecology Section, Institute of Zoology and Botany, University of Tartu, Riia 181, EE-2400 Tartu, Estonia*
[2] *Department of Zoology, Uppsala University, Villavägen 9, S-752 36 Uppsala, Sweden*

SUMMARY

Reproduction and maintenance compete for resources within a single individual. But do individuals invest in reproduction just as much as remains after the requirements of maintenance are covered, or do they sacrifice their health for the sake of still further increase the investment in current reproduction? This question has been found hard to answer because of difficulties of demonstrating that individuals naturally make a reproductive effort of such a magnitude as to inflict health damage. In this paper we present evidence for a trade-off between reproductive effort and health state in great tits, indicated by a positive correlation between total prefledging brood weight and both intensity of *Haemoproteus* blood parasite infection and heterophile:lymphocyte (H:L) ratio. H:L ratios, which signal stress in birds, were high both among individuals making an intense reproductive effort and among aberrantly behaving individuals, such as females incubating in empty nests and birds which abandoned their broods after blood sampling. Experimental reduction of clutch size resulted in decreased intensity of *Haemoproteus* parasitemia, providing further evidence that individual great tits accept immunosuppression to increase their reproductive investment.

1. INTRODUCTION

The concept that reproduction is costly is the basic assumption of life-history theory (Williams 1966; Stearns 1989, 1992), which has been developed to explain inter-populational and inter-individual variations in resource allocation between somatic growth, reproduction, and maintenance in terms of genetically determined evolutionary trade-offs, reflecting different life-history strategies and tactics. Evolutionary trade-offs are based on physiological trade-offs, which implies competition between reproduction and maintenance for energy reserves within a single individual. Individuals behave optimally when they manage to divide their resources between various demands so that their lifetime reproductive output is maximized. Theory predicts that individuals with a high probability of surviving until the next reproductive occasion should not make too exhausting a reproductive effort. However, when their probability of breeding again in the future is low, an optimal reproductive decision may involve allocation of all available resources, including those what would be otherwise used for maintenance, to reproduction. Such 'terminal reproductive investment' is expected to occur if an individual's expectation of future reproduction is low, for example because of old age (Clutton-Brock 1984; Pärt *et al.* 1992), and/or infestation with diseases and/or parasites (Minchella & Loverde 1981; Forbes 1993).

Terminal investment is difficult to observe because individuals whose future reproductive expectations have decreased for whatever reason are likely to be in so poor condition that they cannot make an intense reproductive effort (van Noordwijk & de Jong 1986; Pärt *et al.* 1992). Moreover, experimental demonstration of terminal investment is complicated because one has to show that individuals naturally make such an intense reproductive effort as to put their health at peril. However, a promising new opportunity to tackle this problem is currently developing since it has proven possible to assess quantitatively the health state of individuals and compare it with their reproductive effort under field conditions (Gustafsson *et al.* 1994; Allander 1995).

In this paper we test whether individuals naturally make a reproductive effort of such a magnitude as to cause health damage by examining the relation between reproductive effort, infestation with blood parasites and leukocyte profiles in two populations of the great tit (*Parus major*), breeding under contrasting environmental conditions in rural and urban habitats in south-east Estonia.

The great tit is a small (*ca.* 19 g) short-lived passerine bird, common throughout the Eurasian continent. Some great tits manage to survive and breed in several consecutive years, but more than half of the individuals only breed once during their lifetime. Rural great tits, breeding in mixed deciduous woodland in the vicinity of Tartu, have been found to make an extremely high reproductive effort, as indicated by their average clutch size (11.1 eggs), which is among the highest recorded for the species (Hõrak *et al.* 1995). Moreover, this intense reproductive effort imposes an inter-annual survival cost on the parents, as females who rear all or

* Author to whom correspondence should be addressed.

most of their nestlings to fledging survive worse than females with high nestling mortality (Hõrak 1995). Such a situation has been rarely documented in birds (Lindén & Møller 1989; Partridge 1989), and it provides a unique opportunity to study the mechanisms relating reproductive effort with reproductive costs. Urban great tits, breeding in the town of Tartu, are known to differ from their rural conspecifics by higher adult survival (Hõrak & Lebreton 1996), smaller average clutch size (8.8 eggs; Hõrak *et al.* 1995), higher nestling mortality (Hõrak 1993) and lack of any relation between nestling mortality and parental survival (Hõrak 1995).

Blood parasites (haematozoa) are protozoans living in tissues and in the peripheral blood of their hosts, consuming a variety of host metabolites and haemoglobin. Leukocytes are part of the immune system, and the number and proportions of different types of leukocytes reflect the health state of the individual. The typical response to various stressors (including infectious diseases, parasitemia, social–psychical disruption, and starvation) in birds is a progressive increase in the heterophile:lymphocyte (H:L) ratio in the peripheral blood stream (Gross & Siegel 1983; Beuving *et al.* 1989; Maxwell 1993).

If natural reproductive effort leads to health damage, for example, by increasing the susceptibility of individuals to infection, we will expect a positive correlation between parameters of reproductive effort and parasitemia. We predict that in this case both intensity and prevalence of parasitemia and H:L ratios will be higher among individuals with high total fledgling mass of the brood. We also predict that experimental reduction of brood size will result in reduced parasitemia and H:L ratios. Alternatively, if the reproductive decisions of individuals do not involve a decline in health condition for the sake of current reproduction, the prediction will be that no correlations should exist between the parameters of reproductive effort, parasitemia and H:L ratio. In addition, experimental reduction of brood size in this case should not affect the health state of the individuals.

2. METHODS

During the breeding season of 1995, blood parasites and leukocyte profiles were examined in two great tit populations, breeding in nestboxes in and near the town of Tartu, southeast Estonia (58° 22′ N, 26° 43′ E). The distance between the two study areas was 8 km. In approximately half of the nests, the size of the first clutch was experimentally reduced by removing two eggs on the 7th day of incubation. Also broods where more than one egg did not hatch were included in the category of experimentally decreased clutches. Manipulated clutches resulted in significantly lower numbers of fledglings (6.2 ± 2.2 versus 8.3 ± 2.3; $t_{34,46} = 4.2$, $p < 0.001$) and total prefledging brood weight (108.9 ± 40.2 g versus 138.0 ± 37.8 g; $t_{34,46} = 3.4$, $p = 0.001$) than controls, but initial clutch size and laying date did not differ between the two categories (10.1 ± 1.9 eggs versus 10.1 ± 2.0 eggs; $t_{34,46} = 0.1$, $p = 0.911$ and 29.4 ± 5.3 April versus 28.6 ± 4.1 April; $t_{34,46} = -0.7$, $p = 0.486$). Parents were captured in the nestboxes while feeding 7–9 day old nestlings.

Blood samples of breeding adults were taken from the tarsal or brachial vein to identify and count blood parasites and leukocytes. Each blood sample was smeared at two individually marked microscope slides, air-dried, fixed in absolute methanol, and stained with the Giemsa stain. Slides were examined for presence and abundance of blood parasites (number per 100 microscope fields) under 400× magnification for *Trypanosoma* and *Leucocytozoon*, and under 1000× magnification with oil immersion for *Haemoproteus* and *Plasmodium*. After scanning for blood parasites, 71 slides were picked for estimation of proportion of different types of leukocytes. Slides were chosen randomly with the exception that all aberrantly behaving individuals (four birds which abandoned their broods after blood sampling and three females incubating in empty nests) and most of the intensively parasitized birds were included. The proportion of different types of leukocytes was assessed on basis of an examination of a total of 100 leukocytes under oil immersion. The person examining the blood smears (I.O.) had no information about the individual birds except ring number.

Haemoproteus sp. was the only common blood parasite, occurring in 54% of the individuals (110:203), and therefore we confined our examination of the relation between parasitemia and reproductive effort to this genus. Prevalence of parasitemia is defined as the proportion of infested individuals in a sample, and an individual was considered to be infested when containing more than one *Haemoproteus* per 100 microscope fields. Intensity of parasitemia is expressed as number of *Haemoproteus*/100 fields. In statistical tests, intensity of parasitemia was log transformed to obtain normal distribution, allowing the use of parametric statistical procedures. Prevalence and intensity of parasitemia were combined into one measurement, expressed as log (1 + number of *Haemoproteus*/100 fields), thus scoring zero values for uninfested individuals (see Sundberg 1995). Because females and males did not differ with respect to prevalence and intensity of parasitemia and H:L ratios ($p > 0.5$), and there was no correlation between parasitemia and H:L ratios of breeding partners ($p > 0.6$), we pooled the data for the two sexes in some analyses.

Total prefledging brood mass was used as an indirect measurement of the amount of parental reproductive effort. This measure was obtained by weighing the nestlings 15 d after hatching. As a matter of fact, using fledgling number as measure of reproductive effort yielded virtually identical results.

Parametric statistical procedures (Pearson correlation and Student's *t*-tests) were used except when the assumption of normality of data distribution was violated. In that case nonparametric tests (Mann-Whitney U-test and Spearman rank correlation) were used. Significance levels refer to two-tailed tests, except when decrease of parasitemia in response to manipulation was expected. Data values are reported as mean ± s.d. In the analyses of the relation between reproductive effort, parasitemia and H:L ratio, only data from genuine first clutches which fledged at least one young were included.

3. RESULTS

(*a*) *Reproductive effort and parasitemia*

Experimental reduction of clutch size resulted in lower parasitemia among rural females (log (1 + *Haemoproteus* load) = 2.23 ± 1.82 versus 3.53 ± 1.60; $z_{14,14} = -1.84$, one-tailed $p = 0.033$; Mann-Whitney U-test). The trend was similar when females with unhatched eggs were excluded from the experimental group (2.72 ± 1.67 versus 3.53 ± 1.60; $z_{10,14} = -1.25$, one-tailed $p = 0.106$). In pooled data and other sex-

Table 1. *Correlations between parasitemia and reproductive effort, measured as total prefledging brood weight in great tits*

(Spearman rank correlations (r_s) with log (1 + *Haemoproteus* load) are used for all birds, including noninfested ones. Pearson correlations (r) with log (*Haemoproteus* load) are used for infested individuals only. Data from all first clutches which fledged at least one young, pooled over rural and urban populations.)

all females + males	$r_{s150} = 0.24$, $p = 0.002$
infested females + males	$r_{89} = 0.19$, $p = 0.076$
all females	$r_{s78} = 0.23$, $p = 0.043$
infested females	$r_{45} = 0.25$, $p = 0.093$
all males	$r_{s70} = 0.26$, $p = 0.027$
infested males	$r_{41} = 0.10$, $p = 0.528$

habitat categories the parasitemia was not related to manipulation, regardless whether intensity or prevalence or the combination of the two were compared.

Intense reproductive effort was found to be accompanied by high parasitemia when data were pooled over the two populations (see table 1). The relation held for females and males separately, as well as for pooled data when noninfested individuals were included in the calculations. For females and both sexes combined, the correlations were nearly significant also when only infested individuals were considered. When urban and rural populations and both sexes were analysed separately, the correlations between parasitemia and reproductive effort were not significant below the 5% level. Pooling the data for females and males resulted in a significant correlation between intensity of infection and total brood weight in the rural population ($r_{42} = 0.30$, $p = 0.050$, only parasitised individuals), but not among urban birds.

(*b*) *Reproductive effort and heterophile:lymphocyte ratio*

Parents with experimentally reduced clutches had lower average H:L ratio than parents of control broods when data were pooled over sexes and populations (0.76 ± 0.51 versus 1.05 ± 0.73), but this difference was not significant below 5% level in a two-tailed test ($z_{27.24} = -1.66$, $p = 0.094$; Mann-Whitney U-test). When birds with unhatched eggs were excluded from the experimental group, the result was similar (0.66 ± 0.37 versus 1.05 ± 0.73; $z_{16,24} = -1.81$, $p = 0.071$). No effect of manipulation on H:L ratio was detected when sexes and populations were examined separately.

The highest H:L ratios occurred among individuals making the greatest reproductive effort (see table 2, figure 1). When data were pooled over both sexes, the correlations between H:L ratio and total brood weight were significant irrespective of whether manipulated broods were included in the calculations or not. When sexes were examined separately, only unmanipulated males revealed a significant correlation between reproductive effort and H:L ratio, the correlations for females being on the borderline of significance (see table 2). None of correlations was significant at 5% level when urban and rural populations were analysed separately.

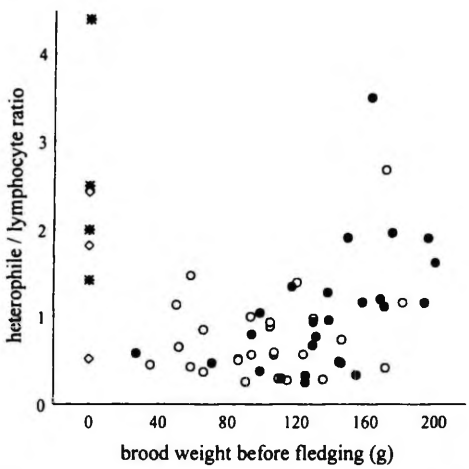

Figure 1. Relation between H:L ratio and reproductive effort, measured as total prefledging brood weight in great tits. Correlation coefficients:significance levels are shown in table 2. Key to figure: open circles = reduced broods; filled circles = control broods; diamonds = females incubating in empty nest; asterisks = birds that deserted broods after blood sampling. The individual with highest H:L ratio (upper left corner) had a freshly broken leg.

All four great tits which abandoned their broods after blood sampling had significantly higher average H:L ratio than the rest of birds (2.57 ± 1.29 versus 0.88 ± 0.63; $z_{3,66} = -2.97$, $p = 0.003$; Mann-Whitney U-test). Also two of three females incubating in empty nests had remarkably high H:L ratios (see figure 1).

We found no significant correlations between H:L ratio and intensity of *Haemoproteus* infection, but the average H:L ratio tended to be higher among noninfested birds (0.83 ± 0.72 versus 1.09 ± 0.81; $z_{29,40} = -1.97$, $p = 0.061$; Mann-Whitney U-test, data pooled over sexes and habitats). This difference became significant below the 5% level in an analysis incorporat-

Table 2. *Spearman rank correlations (r_s) between H:L ratio and reproductive effort, measured as total prefledging brood weight in great tits*

Data from first clutches which fledged at least one young, pooled over rural and urban populations.)

	unmanipulated	all broods
females + males	$r_{s23} = 0.62$, $p < 0.001$	$r_{s51} = 0.41$, $p = 0.002$
females	$r_{s12} = 0.50$, $p = 0.064$	$r_{s29} = 0.34$, $p = 0.064$
males	$r_{s9} = 0.64$, $p = 0.035$	$r_{s20} = 0.42$, $p = 0.051$

ing only urban birds (0.62 ± 0.40 versus 1.03 ± 0.78; $z_{16,34} = -2.08, p = 0.037$; Mann-Whitney U-test, data pooled over sexes).

4. DISCUSSION

Our results demonstrate a trade-off between reproductive effort and health condition, because parents whose investment resulted in high prefledging brood mass suffered higher intensities of parasitemia and elevated H:L ratios, as compared to parents having made a lighter investment. Earlier studies of the great tit have found an increase in parasitemia in response to artificial brood enlargement (Norris *et al.* 1994; Allander 1995; Richner *et al.* 1995). Our study, however, provides the first evidence of birds naturally making a reproductive effort of such a magnitude as to inflict health disorder.

The most likely mechanism for the positive correlation between *Haemoproteus* infection intensity and brood weight is a relapse of a chronic latent infection of parasites as a result of a physiological and environmental stress loading on the immune system imposed by the reproductive effort (Weatherhead & Bennett 1991; Atkinson & van Riper 1991). Elevated H:L ratios of birds are usually associated with increased release of immunosuppressive glycocorticoids, such as corticosterone, which are crucial for survival during severe disruption of homeostasis (Sapolsky 1992) and may assist an organism to reduce an inflammation (Dohms & Metz 1991), but might be detrimental to the health and well-being of an animal during chronic exposure (Zulkifli *et al.* 1994). In poultry the H:L ratios have been shown to increase in response to viral, bacterial, protozoan and fungal infection, but also to other environmental and physiological stressors like various poisons, unbalanced diet, food and water restriction, electric shock treatment, social:psychical disruption, and noise (Gross & Siegel 1983; Dein 1986; Mandal *et al.* 1986; Beuving *et al.* 1989; McFarlane *et al.* 1989; Dohms & Metz 1991; Klasing 1991; Maxwell 1993; Zulkifli *et al.* 1994). We have reason to expect that the high H:L ratios recorded in some of our great tits were associated with stress because, in addition to birds with high reproductive effort, also the aberrantly behaving individuals, such as females incubating in empty nests and birds which abandoned their broods after blood sampling, had distinctively high H:L ratios. As the time from capturing the bird to blood sampling was evidently too short to cause changes in blood profiles, we can exclude the possibility that elevated H:L ratios resulted from handling of birds during capture. Notably, the bird with highest H:L ratio in our study had a broken leg (figure 1).

Our result that H:L ratio and *Haemoproteus* infection intensity did not correlate positively is similar to that of another great tit study (Dufva & Allander 1995). However, noninfested individuals tended to reveal higher H:L ratios in our case. Because the number of leukocytes in blood is a function of infection, the ability to fight infection, and the numbers remaining after the immune system interacts with pathogens (see, for example, Dufva & Allander 1995), it is possible that elevated H–L ratios of noninfested individuals resulted from stress associated with elevated mobilization of immune system. An alternative explanation would be that infection with *Haemoproteus*, which is believed to be a relatively mild parasite (see, for example, Atkinson & van Riper 1991) did not harm their hosts detectably. Interpretation of the relation between parasitemia and health state is further complicated because individuals may appear unparasitised not only because their immune system has suppressed infection, but also because they have (occasionally) not been infested by vectors (blood-sucking *Diptera*).

The results that experimental reduction of clutch size resulted in decreased parasitemia only among rural females, and that only rural birds revealed significant correlation between the intensity of parasitemia and brood weight when the populations were examined separately, are notable in the context of the exceptionally large clutch size (equals 11.1 eggs; Hõrak *et al.* 1995) and low adult survival (equals 0.32; Hõrak & Lebreton 1996) characterizing this great tit population. Great tits cannot exactly predict the food situation for their nestlings at the time of egg-laying, and encounter difficulties in reducing their brood to an optimal size if food for the nestlings proves unexpectedly short (Hõrak 1995). Therefore they may have to face a choice of whether to invest all possible resources to brood rearing to avoid a total failure of the current breeding enterprise, or, alternatively, to invest in the nestlings only that portion of resources that remain after the requirements of their own maintenance have been met. It is possible, therefore, that an optimal decision involves reducing reproductive investment in a situation when the effort required for successful rearing of the brood is so great that it cannot be covered even at the cost of the parent's own maintenance. However, in the situation when the critical amount of resource for successful brood rearing can be reallocated by decreasing the investment in maintenance, it may pay to make a terminal investment because the chances to survive until the next breeding season are low anyway.

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VI

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Health state and local survival in the Great Tit

PEETER HÕRAK, INDREK OTS

SUMMARY - The basic tenet of life-history theory is that reproduction incurs costs in terms of future survival and fecundity of individuals. To examine the physiological mechanisms causing reproductive costs, one has to detect the changes in the physiology of individuals caused by reproductive effort and to test whether the variation in respect to physiological state of individuals has any fitness consequences. In this paper we analyze the relationship between eleven, mostly hemato-serological parental health state indices, measured in the middle of nestling period, and local survival in the Great Tits (*Parus major* L.). The birds were sampled during the breeding periods of 1995 and 1996 in south-east Estonia and their recapture rates recorded in 1996 and 1997. None of the non-parasitological condition indices that responded to brood size manipulations in our previous studies (lymphocyte count in the peripheral blood, heterophile/lymphocyte ratio and hematocrit) was associated with local survival of parents, suggesting that these traits reflected only transient changes in individual's physiology which were probably overridden by the pre- or postmanipulative differences of health state. The only condition index that was associated with local survival rates in our study was the prevalence of *Haemoproteus* blood parasite in yearling Great Tits. 33 % of the uninfected yearlings were recaptured as breeders in the following years but only 12% of infected yearlings were recaptured. Individuals, older than one year showed no relationship between the parasite prevalence and recapture rate. Our results suggest that reproductive costs are mediated by immune function and support the hypothesis that pathogenicity of blood parasites emerges in the age-dependent manner.

RIASSUNTO -

INTRODUCTION

Life-history theory views the reproductive traits as having been adjusted by evolution to optimize individual fitness. The cornerstone of the theory is the concept of trade-offs, representing the costs paid in the currency of fitness when a beneficial change in one trait is linked to detrimental change in another (Stearns 1989).

Although the theory predicts negative correlations between life-history traits, attempts to demonstrate trade-offs often fail at the level of individuals within population due to the confounding effects of individual phenotypic quality. Because individuals differ in their ability of acquisition of resources, some can afford to spend much on several life-history traits while others can spend little, which results in the positive phenotypic correlations between life-history traits overriding the negative ones (van Noordwijk & de Jong 1986). For building the realistic models of life-history evolution and testing their predictions, the concept of individual phenotypic quality, condition or state is therefore indispensable. Even supposing that the opinions of different authors about what constitutes the individual's condition diverge (see *e.g.* Winkler & Allen 1996), it seems generally accepted that it can be considered as a property of an individual which has a potential to affect both the expression of life-history traits and fitness (Price *et al.* 1988, Møller 1991, Schluter & Gustafsson 1993, Hõrak *et al.* 1997).

A great step towards understanding the role of individual condition in life-history evolution has been made by the development of the immunological ecology (Gustafsson *et al.* 1994, Lochmiller 1996, Nordling 1996, Sheldon & Verhulst 1996, Møller 1997). Because immune function interacts with the general health state of an organism and competes for the resources that can be allocated to other activities, immunological research may appear powerful tool to explain why and how do individuals differ from each other in respect to their condition and what are the fitness consequences for these differences. Specifically, the application of various health state assays in combination with manipulations of reproductive effort in wild animal populations has a potential to reveal changes in the physiology of individuals caused by reproductive effort and thus, to detect the potential physiological costs of reproduction. Combining such studies with analysis of survival during the subsequent breeding seasons enables to

test whether the variation in respect of physiological state of individuals can be associated with survival costs of reproduction.

Aim of this study is to describe the relationship between eleven, mostly hemato-serological parental health state indices and local survival in two Great Tit populations breeding in different habitats. Traits examined include plasma protein concentrations, estimates for total and differential leukocyte counts, blood parasitemia, hematocrit and parental body mass. Of these, total plasma protein and albumin levels are potentially interesting because they are known to decrease in almost any pathological state (with concurrent increase of the relative proportion of plasma globulins). Leukocytes form the essential part of the immune system and their number and differential counts (heterophile/lymphocyte ratios) are informative in respect to stress syndrome. The immunocompetence of an individual is also expected to be reflected by its parasite burden, and therefore, we test for the association between recapture rate and intensity and prevalence of *Haemoproteus* blood parasite infection. Additionally, we examine the hematocrit (relative amount of erythrocytes in total blood volume), which is known to decrease due to certain diseases but increase in response to physical exercise. We also test for the relationship between local survival and body mass, which is the most non-specific but most easily obtainable index of physiological state.

Our previous studies (Ots & Hõrak 1996, Hõrak *et al.* 1998) have described the response of health state indices to clutch and brood size manipulations in the same Great Tit populations. During the breeding season of 1995, Ots & Hõrak (1996) recorded the leukocyte profiles and hemoparasite counts in the middle of the nestling period and examined the response of these parameters to experimental reduction of clutch size. They showed that reduction of clutch size resulted in decreased intensity of *Haemoproteus* parasitemia among female Great Tits in one of two study areas. Intense reproductive effort was accompanied by elevated heterophile/lymphocyte (H/L) ratios, suggesting a connection between reproductive effort and heightened stress levels. In 1996, Hõrak *et al.* (1998) found that brood enlargement resulted in elevated H/L ratios and decreased lymphocyte concentration in the peripheral blood, suggesting that increased reproductive effort can be associated with immunosuppression. Also, hematocrit increased in response to brood enlargement, suggesting a response to the requirement of elevated oxygen carrying capacity during increased work load. Parental body mass revealed a tendency (albeit not significant at 5% level) to decrease in response to brood enlargement. No effect of brood size manipulation on total leukocyte count, heterophile count, intensity of *Haemoproteus* parasitemia, or plasma proteins could be detected.

In what follows, we test whether the health state indices examined by us in the previous studies are associated with local survival i.e., the probability of an individual to be recaptured as a breeder in the subsequent years.

METHODS

Data were collected during the breeding periods of 1995 and 1996 in two neighbouring (urban and rural) Great Tit populations breeding in nestboxes in and near the city of Tartu, south-east Estonia (58° 22' N, 26° 43' E). The study areas are described in Hõrak *et al.* (1995).

Adult Great Tits were captured on their nests when nestlings were 8 days old. Birds were weighed with a Pesola spring balance with a precision of 0.1 g and their tarsi measured with a sliding caliper to the nearest 0.1 mm by the same person (PH). To reduce the influence of structural size and diurnal variation, body mass was expressed as the residual from a multiple linear regression of mass on cubed tarsus length and weighing time (measured from sunrise).

Blood samples (100 - 150 µl) for hematological measurements were taken from the tarsal or brachial vein. For identification of blood parasites and leukocytes, a drop of blood was smeared on two individually marked microscope slides, air-dried, fixed in absolute methanol, and stained with azure-eosin. Slides were examined for the presence and abundance of blood parasites under 400× magnification for *Trypanosoma*, Microfilaria and *Leucocytozoon*, and under 1000× with oil immersion for *Haemoproteus* and *Plasmodium*. Scanning of a single slide for parasites took approximately 15 minutes. Because *Haemoproteus* sp. was the only common blood parasite we confined our examination of intensity of parasitemia to this genus.

After scanning for blood parasites, slides were used to estimate total number and proportion of different types of leukocytes. The proportion of different types of leukocytes was assessed on the basis of an examination of a total of 100 leukocytes under oil immersion. Estimates of the total white blood cell count (WBC) and intensity of

parasitemia were obtained by counting the number of leukocytes or parasites per approximately 10000 erythrocytes. Differential leukocyte counts were obtained by multiplying their proportions with WBC. In the analyses, only data for lymphocytes and heterophils as the most numerous immune cells are used. The persons examining blood samples had no information about the individual birds except ring number.

Plasma samples for protein electrophoresis were obtained by centrifuging blood for 10 minutes at 3000 rpm, after which the plasma was stored at -20°C until analysed. 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 densitometrically scanned at 525 ηm wave length. Total plasma protein concentration was determined in a photometric colorimetric test using the Biuret method. Because of difficulties of separating the prealbumin fraction from albumin, summed concentrations for both are reported and termed albumin concentration.

Relationship between condition indices and local survival rates were analysed by comparison of trait values of recaptured ("survivors") and not recaptured ("non-survivors") individuals by t-tests (in case of normally distributed data values) or U-tests (when assumption of normality of data was violated) and with logistic regression (SAS CATMOD procedure; SAS Institute 1985). To avoid pseudoreplication, a test sample was formed for analysing the pooled data. It included birds that were captured only once and randomly picked observations of individuals that were captured in both study years, so that none of individuals was entered more than once in the analysis. No individuals were excluded when data of both study years were analysed separately.

All significance levels refer to two-tailed tests. Data values are reported as mean ± SD. Only data from genuine first clutches were used. Females incubating in empty nests were excluded from all analyses because they were known to differ from the 'normal' birds in respect of hematological parameters (Ots & Hõrak 1996).

RESULTS

Comparison of locally surviving and non-surviving individuals revealed no survival differences between brood manipulation, sex, habitat and age categories, neither when data of 1995 and 1996 were considered separately (all p values >0.1) nor in the data pooled over both study years (all p values >0.3). For comparison of health state indices of survivors and non-survivors, data were therefore pooled over sex, habitat and age categories. As shown in the Table 1, none of the differences was significant at 5% level. In 1995, there was a tendency for heavier individuals to survive better, while in 1996, surviving birds had lower prevalence of *Haemoproteus*. However, none of these tendencies was consistent in two years, neither did the differences became significant when data were pooled over two years.

For the traits that had been measured in both 1995 and 1996, we ran the analysis in the pooled data set and tested for the site, sex and age-specific differences in respect to condition indices and local survival. None of the trait*site or trait*sex interaction terms was significant at 5% level in a logit analysis. However, the relationship between local survival and the prevalence of *Haemoproteus* parasitemia revealed an age-dependent pattern (Table 2). This was because noninfected yearlings survived better than infected ones, while among the older birds, the local survival was not associated with prevalence of *Haemoproteus* blood parasite (Fig. 1). The intensity of *Haemoproteus* parasitemia was not related to local survival neither among yearlings (survivors: 53±310, N=8; non-survivors: 141±310, N=49, z=-1.26, p=0.206) nor among older birds (survivors: 46±52, N=22; non-survivors: 37±43, N=72, z=1.30, p=0.195; Mann-Whitney U-tests on infected individuals only).

DISCUSSION

The concept of reproductive costs is based on the assumption that effort devoted to the current reproduction may reduce individual's chances to survive and reproduce successfully in the future. This assumption has a corollary that reproductive effort of different magnitude has a differential impact on the physiology of an individual. Recently, avian ecologists have started to detect this impact on the basis of simple clinical and parasitological health state indices. To put this knowledge into ecological context, one has to establish the association between the health state indices sensitive to the manipulation of reproductive effort and fitness prospects of an

individual. Testing for such an association was the main goal of the present study.

Our results are negative in respect to non-parasitological health state indices, as none of these revealed significant association with local survival (Table 1). This suggests that traits that appeared most sensitive to brood size manipulation in our previous studies (Ots & Hõrak 1996, Hõrak *et al.* 1998), namely the lymphocyte concentration in the peripheral blood, heterophile/lymphocyte ratio and hematocrit, reflected only transient changes in individual's physiology which were probably overridden by the pre- or postmanipulative differences of health state. This result is perhaps not surprising, given that these indices revealed only moderate or lack of persistency in time. We have shown previously (Ots *et al.* 1998) that the values of lymphocyte count and H/L ratio in adult Great Tits in the fifteenth day of nestling period were only moderately correlated with corresponding eighth day values (r=0.49 and 0.33 respectively), while hematocrit did not reveal any individual constancy during the nestling period. Thus, the state indices that were affected by our brood size manipulation experiment could be hardly considered to reflect any persistent component of individual condition.

Leukocyte data, recorded in this study are difficult to compare with the published record because of the limited number of analogous surveys available. Nevertheless, we are aware of two studies that, contrary to our results, have established the relationship between survival and leukocyte counts or profiles. Wilson and Wilson (1978) showed that captive Red Grouse (*Lagopus lagopus scoticus*) that died after experimental infestation with caecal threadworm had higher heterophile counts in the peripheral blood than birds that survived the threadworm challenge. Bradley *et al.* (1988) showed that Common Rock Rats (*Zyzomys argurus*) that survived the most stressful season had lower neutrophile/lymphocyte ratios (mammalian analogue to avian H/L ratio) than non-survivors. Both authors interpret their results as indicative of lower stress burden of survivors as compared to non-surviving individuals. These results indicate that the absence of relationship between blood profiles and survival, as recorded in our study, may not appear ubiquitous.

The only condition index that was associated with local survival rates in our study was the prevalence of *Haemoproteus* blood parasite infection in yearling Great Tits. 33 % of the uninfected yearlings were recaptured as breeders in the following years but only 12% of infected yearlings were recaptured (Fig. 1). This result can be interpreted in two ways: 1) either birds succumbed due to *Haemoproteus* infection *per se*, or 2) generally low immunocompetence and poor health state of those individuals that were likely to die after reproduction was reflected by the presence of *Haemoproteus* infection as a by-product. The first possibility seems more likely to us because it is compatible with the age-dependent pattern in the association between the prevalence of *Haemoproteus* and local survival. Lack of the effect of infection to local survival in older age classes can be explained by the higher virulence of the parasite in young, immunologically naive birds. Initial infections often appear deadly which makes young age classes most susceptible, but if the host survives, the parasite may enter a chronic phase where host immune responses keep the parasite at low levels with little or no signs of disease (Atkinson & van Riper 1991). It is possible therefore, that most of the adult population carries chronic infections and significant mortality occurs only in the young non-immune cohort. This explanation would be also compatible with the observations that young birds usually possess lower prevalence but higher intensity of blood parasites (Davidar & Morton 1993, Allander & Bennett 1994, Norris *et al* 1994, Merilä *et al.* 1995, Merino & Potti 1995, Sundberg 1995, Ots 1996) that has been ascribed to an acquired immunity (Allander & Bennett 1994) or selective mortality due to genetic resistance to disease (Davidar & Morton 1993). Testing of this hypothesis would require further research of age-specific aspects of parasite induced mortality.

Of previous studies, Davidar and Morton (1993) found no relationship between return rates of Purple Martins (*Progne subis*) and prevalence of *Haemoproteus prognei*, while Dale *et al.* (1996) found that male Pied Flycatchers (*Ficedula hypoleuca*) infected with *Haemoproteus* tended to have higher return rates than noninfected individuals. Weatherhead & Bennett (1992) found no effects of blood parasites (mainly *Leucocytozoon fringillarum* and *Plasmodium vaughani*) on local survival of Brown-headed Cowbirds (*Molothrus ater*) irrespective of age of birds. Analogously, no association between mortality and infection by various blood parasites was found in ten other studies performed in different bird species (reviewed by Bennett *et al.* 1993). To our knowledge, the only study that has

established lower local survival rates in association with Haematozoan infections was that of Richner *et al.* (1995) on male Great Tits infected with *Plasmodium*. However, there was no significant relationship between the infection status and return rates of females in the same population (Oppliger *et al.* 1997).

Does *Haemoproteus* or, blood parasites in general, appear suitable candidates for explaining the physiological mechanism for the reproductive costs? To fulfil this criterion, a trait has to 1) depend on reproductive effort and 2) incur costs in terms of hosts future survival or fecundity.

With some reservations, the first assumption seems generally well supported in avian studies (reviewed by Møller, 1997). The most unambiguous results were obtained by Allander (1997) who demonstrated that both prevalence and intensity of *Haemoproteus* correlated positively with experimental brood size in both female and male Great Tits. In addition, blood parasitemia appears a relatively stable condition index because the intensity of *Haemoproteus* in adult Great Tits revealed the greatest persistency in time during the nestling period when compared to hemato-serological parameters (Ots *et al.* 1998).

At present, it is more difficult to say anything about the validity of the second assumption, because from the studies testing the effect of brood size manipulations on parasitemia, only that of Richner *et al.* (1995) examined (and detected) the effect of parasites on host survival. The question cannot be unambiguously answered on the basis of the current study either, because although our results suggest the pathogenicity of *Haemoproteus* to yearlings, our brood size manipulation experiments did not gave a clear cut results in respect of parasitemia. In 1995 clutch size manipulation affected only intensity but not prevalence of parasitemia among the females in one of two populations (Ots & Hõrak 1996), while in 1996 we failed to detect any effect of brood size manipulations on *Haemoproteus* infection (I.Ots and P. Hõrak, unpublished data). One of the potential reasons for such an inconsistency of manipulative effects on parasites might be the inequality of individuals in respect to exposition to infection. This means that some birds might not respond to increased reproductive effort by increased parasitemia because they have not been (occasionally) infected by the vectors.

We therefore conclude that the present state of the art does not enable to consider the blood parasites as a convenient and robust tool for examination of the mechanisms of reproductive costs. Further studies of suitable non-parasitological condition indices that would be associated with both reproductive effort and individual's general well-being are therefore urgently required. A promising perspective in this area will be the measurement of immunocompetence by challenging birds with novel antigens and subsequent quantification of antibody responses. Few first avian studies have already revealed that immune response to novel antigens can be affected both by the nutritional state and reproductive effort (Deerenberg 1996) and that individuals showing the highest immune response to novel antigen may be more likely to survive until the following breeding season (Saino *et al.* 1997). Combining such studies of immune response with basic clinical tests will enable to check which (and whether at all) simple health state indices, obtained by a single sampling of an individual are suitable for predicting its general well-being and immunocompetence.

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PEETER HÕRAK
Estonian Institute of Zoology and Botany
Riia 181, EE2400, Tartu, Estonia

INDREK OTS
Institute of Zoology and Hydrobiology, Tartu University
Riia 181, EE2400, Tartu, Estonia

Table 1. Comparison of state indices of locally surviving and non-surviving Great Tits. Individuals recaptured as breeders in years following blood sampling are termed as survivors while individuals that were not recaptured later are termed as non-survivors. Tests statistics refer to Mann-Whitney U-tests (z), t-tests (t) and χ^2 -tests (χ^2). Comparison of *Haemoproteus* intensity concerns infected individuals only.

Trait	Year	Mean±SD non-survivors	Mean±SD survivors	Test statistic $_{(DF)}$	p-diff.
Albumin/Globulin ratio	1996	1.90±0.53	1.67±0.41	$z_{(84,25)}$ =-1.83	0.068
Abumin (g/l)	1996	21.3±4.5	20.5±4.7	$z_{(77,22)}$ =-0.89	0.375
Total plasma protein (g/l)	1996	34.4±9.4	32.5±6.1	$z_{(102,38)}$=-0.88	0.379
Hematocrit	1996	0.52±0.05	0.53±0.05	$t_{(94,37)}$ =-1.27	0.206
Total leukocyte count (cells/≈10 000 erythrocytes)	1995	51.8±21.5	31.7±19.9	$z_{(42,11)}$ =-1.40	0.163
	1996	53.7±25.5	54.6±27.2	$z_{(122,47)}$ =-0.01	0.994
H/L ratio	1995	0.89±0.66	0.96±0.57	$z_{(42,11)}$ =-0.65	0.514
	1996	0.91±0.91	0.87±0.51	$z_{(121,47)}$ =-1.40	0.254
Lymphocyte count (cells/≈10 000 erythrocytes)	1995	26.3±12.7	19.3±8.6	$z_{(42,11)}$ =-1.61	0.107
	1996	31.2±17.8	31.5±19.9	$z_{(121,47)}$ =-0.35	0.725
Heterophile count (cells/≈10 000 erythrocytes)	1995	21.7±17.7	17.9±13.8	$z_{(42,11)}$ =-0.75	0.451
	1996	22.1±15.3	22.7±12.9	$z_{(121,47)}$ =-0.77	0.443
Residual body mass	1995	-0.15±0.80	0.09±0.83	$t_{(118,41)}$ =-1.66	0.098
	1996	-0.41±0.82	-0.44±0.89	$t_{(121,48)}$ =0.19	0.851
Intensity of *Haemoproteus* (#/≈10 000 erythrocytes)	1995	76±123	37±145	$z_{(66,25)}$ =-1.37	0.169
	1996	76±260	57±73	$z_{(62,16)}$ =-0.62	0.553
Prevalence of *Haemoproteus* (% of infected individuals)	1995	54.2% (65/120)	61.9% (26/42)	χ^2_1=0.76	0.384
	1996	45.5% (56/123)	31.4% (16/51)	χ^2_1=2.98	0.084

Table 2. Age-specific effect of *Haemoproteus* prevalence on local survival rates of Great Tits. 297 observations, pooled over 1995 and 1996; individuals that were sampled in both years are included only once. Site stands for two (urban and rural study sites) and Age for two age classes (yearlings and older). Probability of likelihood ratio, greater than 0.05 indicates that model fits the data reasonably well.

Trait	DF	χ^2	p
Year	1	0.98	0.321
Site	1	0.91	0.340
Sex	1	0.00	0.948
Age	1	0.07	0.794
Haemoproteus	1	1.58	0.209
Age* *Haemoproteus*	1	8.56	0.003
Likelihood ratio	24	18.48	0.779

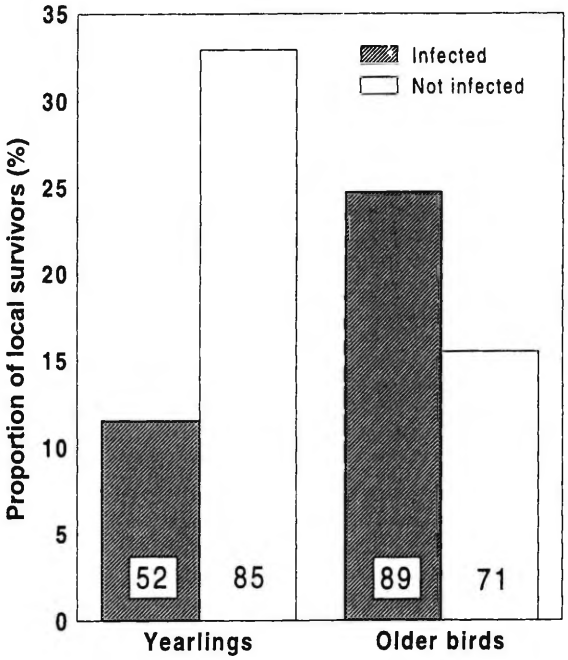

Figure 1. Relationship between local survival and prevalence of *Haemoproteus* in yearling and older Great Tits. Data pooled over 1995 and 1996; individuals that were sampled in both years are included only once. Difference in prevalence between survivors and non-survivors is significant in yearlings (χ^2_1=6.82, p=0.009) but not in older birds (χ^2_1=1.53, p=0.148).

CURRICULUM VITAE

INDREK OTS
Date of birth: November 19, 1972
Languages spoken: Estonian, English, Russian

Education

1991 Ülenurme Secondary school
1996 Tartu University, diploma degree in zoology (*cum laude*)
1997 Tartu University, MSc Hematological health state indices of reproducing Great Tits. Methodology, sources of natural variation and a response to brood size manipulation

Research experience

May 1994 – June 1994 Institute of Zoology and Botany, laboratory assistant
April 1995 – June 1995 Tartu University, Inst. of Zool. and Hydrobiol., laboratory assistant
March 1996 Institute of Zoology and Botany, laboratory assistant
April 1996 – Dec. 1996 Tartu University, Inst. of Zool. and Hydrobiol., laboratory assistant,
Jan. 1997 – Feb. 1999 Institute of Zoology and Botany, laboratory assistant

Research training

Nov. 1995 Institute of Ecology, Vilnius, Lithuania
April 1996 Uppsala University, Department of Zoology
Jan. – March 1997 Uppsala University, Department of Zoology

Main scientific interests

Current research program: Immunological ecology

Honours and Awards

1996 Estonian Academy of Science contest of student works, II prize
1997 Estonian Academy of Science contest of student works, III prize

Grants and Scholarships

Dec. 1996 Scholarship of Rudolf Sõmermaa foundation within the corporatio Ugala in Sweden, 1500 CAD
Jan. 1997 Scholarship of Nordic Council of Ministers, 21000 SEK
1998–1999 Estonian Science Foundation grant for doctoral students, 38209 EEK

Publications

1. Hõrak, P., Mänd, R, and Ots, I. 1995. Egg size variation in the Great Tit: individual, habitat and geographic differences. — *Ornis Fennica* 72: 97–114.
2. Ots, I. and Hõrak, P. 1996 Great tits (*Parus major*) trade health for reproduction. — *Proceedings of the Royal Society of London, Biology*. 263: 1443–1447.
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Presentation of work

1. Hõrak, P. and Ots, I. 1997. Immunological ecology of Great Tits: physiological costs of reproduction. — Abstracts of the 1st meeting of European Ornithological Union (Bologna 28–30 August 1997).
2. Ots, I. and Hõrak, P. 1998. Health impact of blood parasites in breeding Great Tits. (Symposium on ecology of bird-parasite interactions, Vilnius 25–28 June 1998).
3. Hõrak, P. and Ots, I. 1998. Immunological ecology of Great Tits. — Ostrich 69, p. 32 (Proceedings of 22nd International Ornithological Congress, Durban 16–22 August 1998).

CURRICULUM VITAE

INDREK OTS

Sünniaeg: 19. november 1972

Keelteoskus: eesti, inglise, vene

Hariduskäik

1991 Ülenurme Keskkool

1996 Tartu Ülikool, *baccalaureus scientiarum* zooloogia erialal (*cum laude*)

1997 Tartu Ülikool, *MSc* loomaökoloogia erialal (*Hematological health state indices of reproducing Great Tits. Methodology, sources of natural variation and a response to brood size manipulation*)

Teenistuskäik

Mai 1994 – juuni 1994 Zooloogia ja Botaanika Instituut, laborant

Apr. 1995 – juuni 1995 TÜ zooloogia ja hüdrobioloogia instituut, laborant

Märts 1996 – märts 1996 Zooloogia ja Botaanika Instituut, vanemlaborant

Apr. 1996 – dets. 1996 TÜ zooloogia ja hüdrobioloogia instituut, laborant

Jaan. 1997 – dets. 1997 ja veebr. 1998 – veebr. 1999 Zooloogia ja Botaanika Instituut, vanemlaborant

Erialane enesetäiendus

Nov. 1995 Vilniuse Ökoloogia Instituut

Apr. 1996 Uppsala Ülikooli zooloogia kateeder

Jaan. – märts 1997 Uppsala Ülikooli zooloogia kateeder

Tunnustused

1996 Eesti Teaduste Akadeemia üliõpilaste teadustööde konkurss, II preemia

1997 Eesti Teaduste Akadeemia üliõpilaste teadustööde konkurss, III preemia

Teadustegevus

Peamised uurimisvaldkonnad: immunoloogiline ökoloogia

Muu teaduslik-organisatsiooniline ja erialane tegevus: Olen Eesti Looduseuurijate Seltsi, Eesti Ornitoloogiaühingu ja Balti Parasitoloogia Seltsi liige.

Saadud uurimistoetused

dets. 1996 Korporatsioon Ugala Rudolf Sõmermaa nimeline stipendium õpingutoetuseks; 1500 CAD

jaan. – märts 1997 Põhjamaade Ministrite Nõukogu stipendium õppimiseks Uppsala Ülikoolis; 21 000 SEK

1998–1999 ETF grant nr. DBGZH0626 (doktorandi teadustöö stipendium); 38 209 EEK

Teaduslikud publikatsioonid

1. Hõrak, P., Mänd, R, and Ots, I. 1995. Egg size variation in the Great Tit: individual, habitat and geographic differences. — *Ornis Fennica* 72: 97–114.
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Konverentside teesid

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