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Variation in haematological indices and immune function during the annual cycle in the Great Tit *Parus major*

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We investigated seasonal variation in haematological indices and immune function in the non-migratory Great Tit *Parus major* over a complete annual cycle. The haematocrit value showed a marked reduction in spring and summer, reaching a lowest value during moult, after which it increased to reach a maximum in winter and spring. The peak in the heterophil to lymphocyte ratio (H/L) during July indicated that Great Tits were the most stressed during the first half of the moulting period. The increase in heterophils and H/L ratio, concurrent with a reduced number of lymphocytes during the breeding season, probably reflected the cost of reproduction in terms of physiological stress and immune suppression. After breeding the number of heterophils and the H/L ratio decreased, reaching a lowest value during winter. The concentration of immunoglobulins followed the seasonal pattern in the number of heterophils, though highest values occurred somewhat later, in July–September during the second part of the moulting period. Our observations indicated large differences in activity throughout the year of different components of the immune system. This suggests differences in function among the components and possibly differences in susceptibility to stress, parasitism and hormones during the annual cycle. When juveniles became independent of their parents, the immunoglobulin concentration increased, whereas other immune measures did not show a significant change. This indicates a rapid increase of at least one component of the immune system after the young fledge.

Key words: annual cycle, haematocrit, health state, immunity, immunoglobulins, *Parus major*, white blood cells

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Studies on birds and mammals indicate that seasonal variation in haematological variables and immune defence is the rule rather than an exception (Hasselquist *et al.* 1999, Nelson *et al.* 2002, Lozano & Lank 2003, Møller *et al.* 2003, Fair *et al.* 2007, Buehler *et al.* 2008a). However, results are contradictory (e.g. Nelson *et al.* 2002, Møller *et al.* 2003) for multiple reasons. First, the activity of the immune system is frequently assessed by measuring only one aspect, or by using only a restricted number of variables to assess different branches of the defence system (e.g. Gonzalez *et al.* 1999, Hasselquist *et al.* 1999, Christe *et al.* 2002,

Lozano & Lank 2003, Martin *et al.* 2004; but see Roulin *et al.* 2007 and Buehler *et al.* 2008a as exceptions). Immune responses are extremely complex, reflecting the need to protect the host against a plethora of pathogens (Nelson *et al.* 2002). Moreover, trade-offs between different immune branches can arise (Ardia 2007), and an increase in one measure may evoke a reduction in another. Second, the sampling period may be too short or sampling is too infrequent to describe seasonal changes in the physiological condition and immune activity effectively (e.g. Hórak *et al.* 1998a, Gonzalez *et al.* 1999, Hasselquist *et al.* 1999, Christe *et*

al. 2002, Pap 2002, Owen-Ashley *et al.* 2006). In contrast, Buehler *et al.* (2008a) followed the change in concentration and activity of several immune indices of the Red Knot *Calidris canutus*, a long-distance migratory species, during the complete annual cycle. This sets a yardstick with which to compare birds with contrasting life histories. For example, sedentary and migratory species may differ in exposure to environmental stress, like parasitism, energetic expenditure, food supply and temperature, which may result in divergent selection on immune functions during the annual cycle.

Our main objective is to describe seasonal variation in haematological indices and immune function under field conditions in the Great Tit *Parus major*, one of Europe's most common non-migratory species. Understanding the seasonal variation in the haematological indices and immune function of temperate zone birds is important to make a comparison with species with different life histories (see Buehler *et al.* 2008a). Because of the sex-specific energetic, physiological and behavioural costs of maintaining an effective immune system (Sheldon & Verhulst 1996, Lochmiller & Deerenberg 2000), we expect a difference in immune functions between males and females during the annual cycle (Hasselquist 2007, Martin *et al.* 2008). We explore therefore sex effects in the activity of the immune system. In addition, we investigate the way individuals acquire a mature defence system during ontogeny, by recording the haematological variables and immune function of the same individuals throughout the nestling and independence period. We expect that due to the rapid development of Great Tit nestlings, immune measures promoting defence increase rapidly during independence (Buehler *et al.* 2009). We think our observations provide new insights in the development of the immune system in wild birds as most of our knowledge in this field comes from studies on birds in captivity and poultry (Ardia & Schat 2008).

METHODS

Study site, studied species and captures

We studied a local Great Tit population in the surroundings of the village Stana (46°89'N, 23°14'E, Transylvania, central Romania) between April 2004 and March 2006. The study site is situated in a 40 ha orchard of several species of old fruit trees. Pastures and arable fields surround the orchard. Great Tit has an annual cycle typical for most European non-migratory species. It starts breeding during the second part of April, and the nestlings fledge generally until the end of

June. In 2004, Great Tits fledged between 22 May and 6 June, and in 2005 between 18 May and 17 June. After breeding, adult birds perform their annual complete post-breeding moult, which may last several months between June and September (Pap *et al.* 2007). Juveniles, which fledge between June and July, replace some of the remiges and all rectrices, and their body feathers and wing coverts during the moulting period. The temporal dynamics of the partial post-juvenile moult is similar to that of the adults. In September, birds start to form winter flocks, indicating the preparation for the winter (Cramp & Perrins 1993).

During the study period we regularly captured and ringed birds by using mist nets (Ecotone, Poland) which resulted in 455 captures, including 135 recaptures. Age and sex (when possible based on plumage characters) were scored following Svensson's (1992) criteria, where 'juveniles' are birds before their first complete post-breeding moult, and 'adults' are those after the moult. In this way, 'juveniles' include second-year birds, which are reproductively active without having completed moult yet. Sample sizes of the haematological and immune indices are variable, depending on the number of sampled birds. During the winter, we used sunflower as bait to increase the number of captured birds. The bait was provided several days before capturing and we stopped feeding after the capture session. In this way, we minimized the confounding effect of supplemental feeding on the physiology of the birds. At capture we ringed, aged and sexed the birds, and we measured tarsus length, wing length, and body mass.

In the winter of 2004, we mounted 180 nestboxes in the orchard to study the breeding biology of Great Tits. In 2004 and 2005, we followed 14 and 10 successful broods, respectively. We followed the nests by almost daily visits to determine date of clutch initiation, clutch size, hatching date, brood size, fledging date and number of fledglings. In order to study the change in the haematological indices and immune function during ontogeny, we measured the nestlings and took blood samples (as described below) at the age of 15 days, and subsequently after independence we recaptured as many fledglings as possible.

Haematological measures

We collected blood samples (within 20 min after capture to avoid stress-induced immunosuppression; see Buehler *et al.* 2008b) for analyses in a capillary tube (70 μ l) from the brachial vein. A drop of blood was smeared on a slide, air-dried, fixed in absolute methanol, and stained with May-Grünwald and Giemsa. The haematocrit value was obtained by cen-

trifugation of heparinized capillary tubes for 10 min at 10 000 RPM. After the haematocrit was determined, plasma was separated from blood cells and stored at -20°C until analysis. Smears were examined at $1000\times$ magnification and the proportion of different types of leukocytes was assessed on the basis of examination of 100 leukocytes. The number of white blood cells was expressed per approximately 10 000 erythrocytes. We excluded monocytes, eosinophils and basophils from the analyses because of their low concentration (less than 10 cells/10 000 erythrocytes). The counts of the white blood cells were made by the same person, and these proved to be highly repeatable (see Pap 2002). Total leukocyte counts (WBC) and the composition of leukocytes are considered as indicators of the health status, parasitic infestation and stress (Ots *et al.* 1998). Leukocytosis (increase in the number of leukocytes) is most commonly attributed to infectious diseases (Fudge 1989). Heterophils are non-specific phagocytic immune cells, and as parts of the innate immune defence they play an important role during the initial stages of most infections. Increased level of heterophils is most common during inflammation, and it is a non-specific response to foreign invasion or tissue damage. Lymphocytes are highly specific immune cells, and they are the main cell types in the adaptive immune response (i.e. B-cells and T-cells). Decreasing lymphocyte levels may indicate immunosuppression, while they proliferate during infections (Ots *et al.* 1998). The heterophil to lymphocyte ratio (H/L) is widely used as an indicator of stress (Davis *et al.* 2008), and the measure is known to increase under stressful conditions.

Due to an accident, the plasma collected in February 2006 was lost, which left samples of 11 months to analyze for immunoglobulins. Immunoglobulins, being part of the humoral immune system, play an important role in innate and acquired immune response (Roitt *et al.* 1996). Increase in immunoglobulin concentration in the peripheral blood is related to parasite infestations (de Lope *et al.* 1998, Ots & Hřrak 1998, Szép & Møller 1999). Total immunoglobulin includes circulating IgG molecules with a role in first-line defence against pathogens (Tizard 2004). We quantified the immunoglobulin concentration by spectrophotometry (Khokhlova *et al.* 2004). Concentrations as low as 24 mg/l of heavy metal salts precipitate the immunoglobulin, since the electric charge and colloidal stability of gamma globulins are lower than those of serum albumins at pH 7.4. We mixed $3.3\text{ }\mu\text{l}$ of plasma with $196.7\text{ }\mu\text{l}$ of 0.024% barbital buffer zinc sulphate solution and allowed immunoglobulins to precipitate for 30 min at room temperature ($22\text{--}23^{\circ}\text{C}$). Immunoglobulin

concentration expressed in optical density units (ODU) was read by spectrophotometer ($\lambda = 475\text{ nm}$, $d = 0.5\text{ cm}$) (Pap *et al.* 2008).

Statistical analyses

Data were analyzed by fitting linear mixed effect models with individual birds as random factor (the 'lme' function of the R interactive statistical environment; R Development Core Team 2005), thus controlling for the effect of pseudo-replication caused by the recaptures. Because the sex of juveniles was not determined, the effect of age, sex and the interaction was not tested simultaneously in the same model. Therefore, we first fitted a model with month and age as explanatory variables. In a next model, we analyzed the effect of sex of adult birds. Because of the low sample size of adult females in some months we pooled data into three seasons (breeding: March–June, moulting: July–October, wintering: November–February). In this analysis, the denominator degrees of freedom are different for season and sex because season is *inner* to individual, i.e. its value can change within individual (the random effect), while sex is *outer* to individual, i.e. it cannot change within individual (Pinheiro & Bates 2000, p. 91). Both month and age are *inner* to individual, so they have the same degrees of freedom. Data of immunoglobulins, heterophils, H/L ratio were log-transformed to fit the distributional assumptions of the linear models. The difference in immune measures between adult males and females and nestling was tested using one-way ANOVAs with planned comparison of least square means, and Tukey post-hoc tests for unequal sample size was used to test the difference between specific groups. We found no significant difference in morphological and physiological variables of the nestlings born in 2004 and 2005, and therefore the effect of year was omitted in the analyses about the immune function of nestlings and fledglings. Means are presented $\pm\text{SD}$.

RESULTS

Seasonal variation in the haematological indices and immune function

Haematocrit value varied significantly over the annual cycle (Table 1, Fig. 1A), but was not affected by sex or age. Haematocrit decreased during the breeding season, and after reaching a lowest value during the moulting period in August it increased in autumn, reaching a highest value in winter and spring. The significant interaction between month and age indicates a difference between adults and juveniles in the pattern

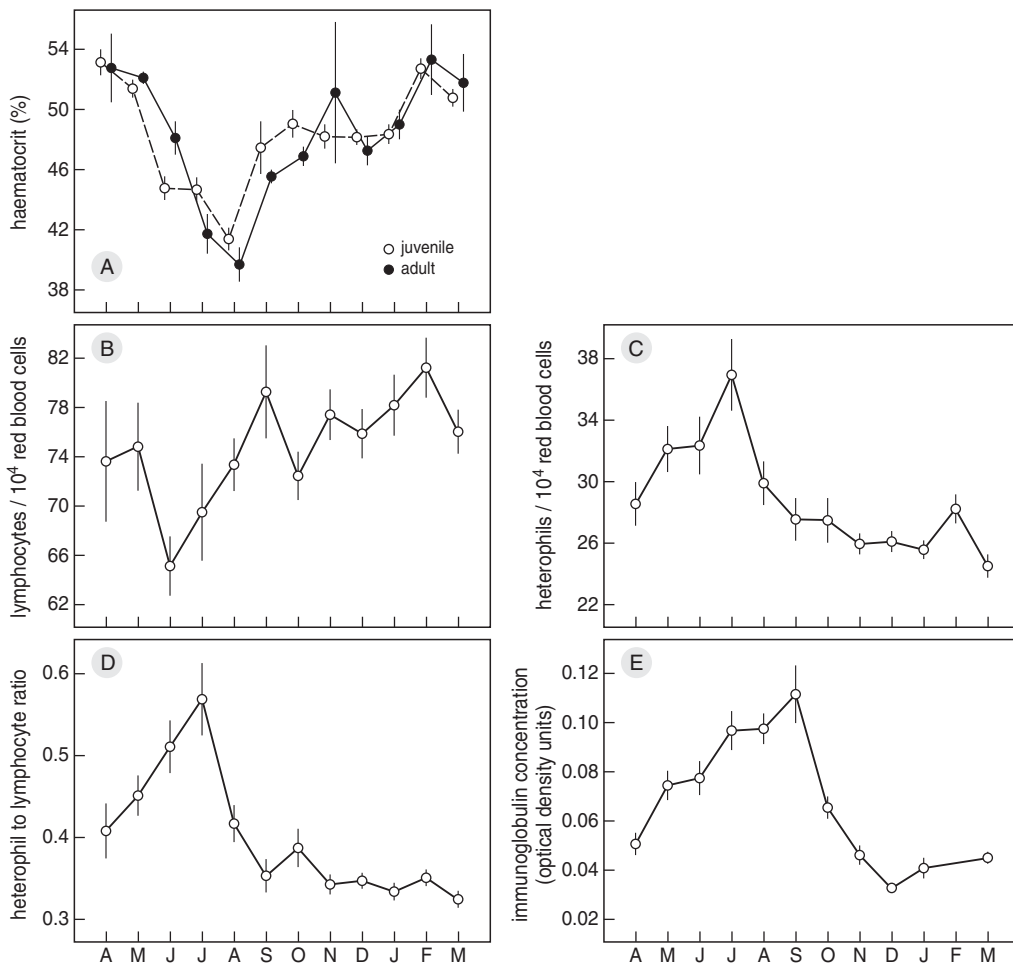


Figure 1. Haematocrit level (A), lymphocyte number (B), heterophil number (C), H/L ratio (D) and total immunoglobulin concentration (E) in the Great Tit during the annual cycle. Indicated are mean values \pm SE. In case of haematocrit, open circles indicate juveniles and closed circles adults.

of haematocrit during the annual cycle. WBC, heterophils, lymphocytes, H/L ratio and immunoglobulin concentration were similar between age classes and sexes (Table 1), also indicated by the absence of significance in the month \times sex and month \times age interactions. WBC did not change significantly during the year (Table 1), while lymphocytes, heterophils and the H/L ratio showed a marked variation through the annual cycle (Table 1, Fig. 1B,C,D). The number of heterophils and H/L increased during the breeding and beginning of the moulting season, from April until July, reaching the highest value during the first half of the moulting season in July. During August to November, when Great Tits perform their annual moult and prepare for the winter, the number of heterophils and the H/L ratio decreased, reaching a lowest value during the winter. The number of lymphocytes showed an opposite pattern

with lowest values during the end of breeding and beginning of moult in June. The immunoglobulin concentration showed significant seasonal variation (Table 1, Fig. 1E) with a marked increase throughout breeding and moulting, and reaching a highest value during the second part of the moulting period in September. Subsequently, immunoglobulin decreased during autumn, and reached a lowest value in winter.

Changes during independence of juveniles

The haematocrit level of nestlings was significantly lower than in parents and the immunoglobulin concentration was significantly higher than in adult females (Table 2; planned comparison between adults and nestlings, haematocrit: $F_{1,64} = 41.88$, $P < 0.0001$, immunoglobulins: Tukey post-hoc test for unequal sample size between nestlings and adult females, $P < 0.001$). WBC,

Table 1. Haematocrit level, WBC, heterophil and lymphocyte number, H/L ratio and total immunoglobulin concentration in the Great Tit in relation to month, age and sex of adult birds, by linear mixed effect models (see also Methods).

Adults and juveniles				Adults			
Term	df	F	P	Term	df	F	P
Haematocrit							
Month	11,111	51.01	<0.0001	Season	2,20	44.2	<0.0001
Age	1,111	0.01	0.92	Sex	1,92	0.05	0.82
Month × Age	11,111	2.07	0.03	Season × Sex	2,20	0.63	0.54
WBC							
Month	11,92	1.33	0.22	Season	2,17	0.46	0.64
Age	1,92	0.01	0.94	Sex	1,86	0.14	0.71
Month × Age	11,92	0.92	0.53	Season × Sex	2,17	0.08	0.92
Lymphocyte							
Month	11,92	2.85	<0.01	Season	2,17	0.54	0.59
Age	1,92	0.03	0.86	Sex	1,86	0.28	0.6
Month × Age	11,92	0.88	0.57	Season × Sex	2,17	0.05	0.95
Heterophil							
Month	11,92	4.98	<0.0001	Season	2,17	2.26	0.14
Age	1,92	0.10	0.75	Sex	1,86	2.58	0.11
Month × Age	11,92	1.10	0.37	Season × Sex	2,17	0.29	0.75
H/L ratio							
Month	11,92	8.42	<0.0001	Season	2,17	3.48	0.054
Age	1,92	0.16	0.69	Sex	1,86	2.88	0.09
Month × Age	11,92	1.32	0.23	Season × Sex	2,17	0.18	0.84
Immunoglobulins							
Month	10,84	27.45	<0.0001	Season	2,18	13.15	<0.0001
Age	1,84	0.08	0.78	Sex	1,82	1.34	0.25
Month × Age	10,84	1.42	0.19	Season × Sex	2,18	0.07	0.93

Table 2. One-way ANOVAs comparing haematological and immunological parameters of adult Great Tits during the breeding period and their nestlings (15 days old). Given are means ±SD with sample sizes in parentheses.

Variable	Female	Male	Nestling	F	P
Haematocrit	51.4 ± 2.4 (24)	50.4 ± 2.7 (22)	44.6 ± 5.4 (21)	21.44	<0.0001
WBC	111.6 ± 21.5 (19)	109.9 ± 29.1 (19)	108.2 ± 4.1 (21)	0.14	0.87
Lymphocyte	74.1 ± 20.3 (19)	75.0 ± 19.7 (19)	72.6 ± 3.4 (21)	0.11	0.90
Heterophil	32.3 ± 8.3 (19)	31.6 ± 18.1 (19)	28.8 ± 1.4 (21)	0.54	0.59
H/L ratio	0.46 ± 0.15 (19)	0.43 ± 0.23 (19)	0.40 ± 0.02 (21)	0.85	0.43
Immunoglobulins	0.054 ± 0.020 (24)	0.088 ± 0.041 (22)	0.074 ± 0.013 (21)	9.11	<0.0005

Table 3. Change in the haematological and immunological parameters between the nestling and fledgling stage of individual Great Tits (*n* = 12). Birds were recaptured 62 ± 47.3 days after initial measurements. Paired sample *t*-test, the values are means ±SD.

Variable	Nestling	Fledgling	<i>t</i>	<i>P</i>
Haematocrit	45.9 ± 4.6	44.9 ± 3.2	0.70	0.50
WBC	107.5 ± 4.3	108.1 ± 17.6	−0.13	0.90
Lymphocyte	72.0 ± 3.8	71.9 ± 14.4	0.02	0.98
Heterophil	28.9 ± 1.1	30.4 ± 6.5	−0.74	0.48
H/L ratio	0.40 ± 0.02	0.43 ± 0.12	−1.02	0.33
Immunoglobulins	0.076 ± 0.014	0.098 ± 0.029	−2.41	0.03

heterophils, lymphocytes and the H/L ratio were similar between parents and nestlings. During the 62 ± 47.3 days between the first measure as nestlings and the second measure as fledglings the immunoglobulin concentration increased significantly (Table 3). The haematocrit, WBC, heterophils, lymphocytes and H/L ratio did not change during this period. These results about the haematological indices and immune function during independence could not be confounded by fledging date, since none of the measures were related to fledging date (all $P > 0.4$), which discounts a possibility of confounding date effects.

DISCUSSION

We showed that haematological indices and immune function fluctuate during the annual cycle of the non-migratory Great Tit with large variation in pattern among the measures. This variation probably reflects different functions and susceptibility to stress, parasitism, energy supply or hormones. Some of the changes, as the increased heterophil and immunoglobulins concentration during summer, correspond to what has been called the 'breeding season – high exposure' hypothesis (Hasselquist 2007), i.e. an enhancement of the immune activity during spring and breeding as a result of the adaptive response of the immune system to the seasonally emerging parasites' attacks. On the other hand, the opposite pattern of lymphocytes with high numbers in winter is in favour of the hypothesis of a hormonal and energetical incompatibility of the immune function with reproduction (Hasselquist 2007, Martin *et al.* 2008). Careful experiments about the susceptibility and function of different branches of the immune system would clarify the mechanism responsible for the seasonality of the immune indices that we observed. Our results of differential seasonal patterns among immune functions correspond to the conclusion by Buehler *et al.* (2008a) who arrived at the same conclusion based on observations in the long-distance migratory Red Knot. There is a striking similarity in the pattern of heterophils and lymphocytes between Great Tit and Red Knot, suggesting that some common factors are responsible for mediating the variation of these immune indices in migratory and sedentary birds.

Haematocrit is an indicator of the oxygen carrying capacity of the blood, and increases as a response to elevated metabolic activity. The marked increase during the autumn and winter might be explained by the preparation of birds to the cold winter by enhancing the oxygen carrying capacity of the blood, the increased

locomotor activity of the birds as the moulting season ends, and dehydration (Fair *et al.* 2007). The observed decrease of haematocrit during breeding with a low during moulting is in accordance with the general pattern in birds (Fair *et al.* 2007), and can be explained by the reduced oxygen need of the organism due to increased ambient temperature (Fair *et al.* 2007).

Our observation of an increase in the H/L ratio during the breeding and early moulting period is consistent with previous studies (Gustafsson *et al.* 1994, Christe *et al.* 2002, Pap 2002; but see H  rak *et al.* 1998a). The shift is the result of an increase in the number of heterophils and a concomitant decrease in the number of lymphocytes (see also H  rak *et al.* 1998a) as the breeding season progresses. This increase in the H/L ratio may stem from stressful parental activity during breeding (H  rak *et al.* 1998b, Ilmonen *et al.* 2003). Likewise, the increase in the number of heterophils, and H/L ratio, during breeding can be related to the increase in the number of micro- and macroparasites (e.g. Fudge 1989, Ots *et al.* 1998) as the level of infestation generally increases with a progressing breeding season (e.g. Christe *et al.* 2002, Cosgrove *et al.* 2008).

The drop in number of heterophils and the H/L ratio during moulting up to a low in autumn and winter might be related to a relaxation of energetic stress, a suppression or downregulation of the immune system due to the energetically challenging low temperature (Demas & Nelson 1996, R  berg *et al.* 1998, Svensson *et al.* 1998), or to the absence of parasites during winter (Cosgrove *et al.* 2008). Another interesting pattern in the seasonality of leukocytes is that during summer the number of lymphocytes is low while the heterophils and the concentration of immunoglobulins is raised, followed by an opposite change in the number and concentration of these measures in the subsequent period. This finding may indicate a trade-off between different immune branches suggesting that when one part of the immune system is challenged the other part is down-regulated. Such a trade-off was found in Tree Swallows *Tachycineta bicolor* (Ardia 2007). Individuals that mounted a strong response against phytohaemagglutinin, which stimulates the cellular and innate component of the immune system, had reduced humoral antibody response against sheep red blood cells.

The observed increase in immunoglobulin concentration during breeding is in accordance with previous studies on birds (e.g. Gustafsson *et al.* 1994, Christe *et al.* 2002), but it contradicts the decrease in immunoglobulin concentration between pre-laying and nesting in another Great Tit population (H  rak *et al.* 1998a). Contrary to trends in the number of heterophils and

H/L ratio, immunoglobulins concentration continued to increase until the end of the moulting season in September, after which it dropped to a low during winter. This drop might have a similar basis as the drop in heterophils and H/L ratio in winter as discussed above.

Finally, we found that within a short period of time nestlings acquired a haematological profile and immune function that was close to that of their parents. The number of different leukocytes was similar in nestlings and their parents, while immunoglobulin concentration increased following fledging, reaching the concentration of adults characteristic to this period. Buehler *et al.* (2009) observed similar changes with age in natural antibodies in the Red Knot, suggesting the generality of a rapid ontogeny of the immune system following fledging in birds.

To summarize, we found that (1) components of the immune system exhibit different patterns during the annual cycle, (2) the immune components measured are similar between the sexes, and (3) juveniles obtain an immune profile similar to adults, seemingly reflecting a rapid ontogeny of the immune system. Because the primary function of the immune system is defence, a next step in immunoecological studies should be to determine the role of different components of immunity in protection against specific parasite challenges (Adamo 2004) in order to understand more profoundly the annual cycle of a given immune variable. Further, the cause and consequence of variation of haematological condition and immune function remains to be tested experimentally. Our results about the seasonality of haematological and immunological indices may serve as a framework for further research studying the mechanisms responsible for the variation in physiological condition in birds.

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SAMENVATTING

Het onderhoud van het inwendig afweersysteem van vogels kost energie en bouwstoffen. Vogels bezuinigen hierop zodra dat mogelijk is. Daarom is te verwachten dat de activiteit van het afweersysteem, afhankelijk van de 'afweging' van kosten en baten, aan fluctuaties onderhevig is. Gedurende twee jaren werd in Centraal-Roemenië bij Koolmezen *Parus major* bloed geprikt om te onderzoeken hoe sterk deze fluctuaties zijn. De bloedmonsters werden onderzocht op de hematocrietwaarde (gehalte aan rode bloedcellen), de aantallen van twee typen witte bloedlichaampjes (heterofielen en lymfocyten), de verhouding tussen deze twee (een maat voor de mate van stress waaraan de vogel is blootgesteld) en de concentratie aan afweerstoffen (immunoglobulines). De hematocrietwaarde nam in het voorjaar en de zomer sterk af, bereikte in de nazomer tijdens de rui een dieptepunt en nam daarna weer toe tot een piek in de winter en het voorjaar werd bereikt. De H/L-verhouding (toename aantal heterofielen, afname lymfocyten) was het hoogst in juli, tijdens de eerste helft van de ruiperiode. De toename van de H/L-verhouding in de loop van het broedseizoen is waarschijnlijk een weerspiegeling van de verhoogde stress waaronder de vogels tijdens die periode leefden. Bovendien kan hierbij een rol gespeeld hebben dat de werking van het afweersysteem wordt onderdrukt om voldoende energie vrij te maken voor bijvoorbeeld het voeren van de jongen. Na het broedseizoen keerden de aantallen heterofielen en lymfocyten, evenals de H/L-verhouding weer terug naar het niveau van vóór het broedseizoen. Het gehalte aan immunoglobulines vertoonde een seizoenpatroon dat veel leek op dat aan heterofielen. Uit het onderzoek blijkt dat Koolmezen door het jaar heen een zeer variabel afweersysteem hebben, waarbij grote verschillen in patroon tussen de afzonderlijke onderdelen van het afweersysteem bestaan. De oorzaak van deze verschillen blijft in dit beschrijvend onderzoek duister, maar vermoed wordt dat elke component een eigen gevoeligheid voor stress, parasieten en hormonen heeft. Na het uitvliegen van de jongen veranderde opmerkelijk weinig in de samenstelling van het bloed. Alleen het gehalte van immunoglobulines liet een toename zien. (BIT)

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