

Extremely low ambient temperature affects haematological parameters and body condition in wintering Great Tits (*Parus major*)

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Abstract In high latitudes, thermoregulatory and behavioural adjustments of birds allow survival under extreme ambient temperatures, changing day length, and food availability. In such habitats with such fluctuating environmental conditions, dominant individuals in social species often monopolise safe microhabitats and food resources, which may lead to greater levels of stress in subordinates. The results of this study revealed that certain haematological indices of health state and body condition of wintering Great Tits (*Parus major*) were dependent on their sex and age under conditions of extreme ambient temperature. Heterophil and lymphocyte counts revealed a significant increase in heterophil/lymphocyte ratio (H/L) in female Great Tits during the course of cold spell, where the increase was detected mainly in the first year females. We also found that the condition of pectoral muscle during the cold spells deteriorated only in females, especially in the first year individuals. Since sex appears to be the most

important predictor of the dominance rank and survival in Great Tit winter groups, elevated physiological stress in adult and first year females during cold spells may be explained in terms of increased resource monopolisation by dominant individuals.

Keywords Dominance hierarchy · Cold stress · Health condition · *Parus major* · H/L ratio

Zusammenfassung In großen Höhen machen es Vögeln thermoregulatorische und verhaltensbiologische Anpassungen möglich, unter extremen Umgebungstemperaturen und veränderten Tageslängen und Futterverfügbarkeit zu überleben. In solchen Habitaten mit ihren schwankenden Umgebungsbedingungen monopolisieren innerhalb von sozialen Arten oft dominante Tiere sichere Mikrohabitate und Futterreserven, was bei den subdominanten Artgenossen zu erhöhtem Stress führen kann. Die Ergebnisse dieser Untersuchung machen deutlich, dass bestimmte hämatologische Anzeiger für den Gesundheitsstatus und die körperliche Verfassung überwintender Kohlmeisen (*Parus major*) bei extremen Umgebungstemperaturen von ihrem Alter und ihrem Geschlecht abhängig waren. Zählungen von Heterophilen und Lymphozyten ergaben einen signifikanten Anstieg der Heterophilen/Lymphozyten-Rate (H/L) bei weiblichen Kohlmeisen während Kälteeinbrüchen, wobei dieser Anstieg in erster Linie bei den Weibchen vom gleichen Jahr festgestellt wurde. Wir fanden außerdem, dass sich der Zustand des Herzmuskels während dieser Kälteeinbrüche verschlechterte, allerdings nur bei Weibchen und da auch nur bei denen vom gleichen Jahr. Da bei den Wintergruppen der Kohlmeisen das Geschlecht der wichtigste Prädiktor für Dominanz und Überleben zu sein scheint, kann bei adulten Weibchen vom gleichen Jahr erhöhter Stress während Kälteeinbrüchen möglicherweise

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mit massiverer Ressource-Monopolisierung durch dominante Individuen erklärt werden.

Introduction

In northern Europe, where proper timing of response to seasonal fluctuation of environmental conditions is essential, thermoregulatory and behavioural adjustments of wintering birds allow survival under extremes of ambient temperature, daylength, and food availability (Steen 1958; Chaplin 1976; Prinzinger et al. 1981; Reinertsen and Haftorn 1986). Permanent avian residents in boreal and temperate regions have different strategies for responding to low ambient temperatures (Hissa et al. 1983; Rintamäki et al. 1983). In small birds, the maintenance of high body temperature in cold weather as well as the seasonal improvement of cold tolerance is dependent mainly on heat production, and thus these birds adjust to winter mainly via changes in their metabolism (Dawson and Marsh 1989; Marsh and Dawson 1989).

To survive winters in the higher latitudes, the thermogenic capacity of birds must be sufficient to endure the lowest ambient temperature that may occur. However, it has been demonstrated that different bird species may differ considerably in their capacity for heat production, cold tolerance, heat loss, and ability to reduce their metabolism at night to save energy (Saarela et al. 1995). Because of the higher energy requirements or inability to tolerate reduced body temperature (Reinertsen and Haftorn 1986), wintering individuals may experience greater levels of stress and higher mortality especially under conditions of sudden cold spells.

Vertebrate responses to a non-specific environmental stress are usually manifest in a vast range of physiological changes associated with acute stress (Sapolsky 1992). If the stressor is not removed, an increase of glucocorticoids produces a secondary response associated with chronic stress (Gross and Siegel 1983; Lindström et al. 2005). In the majority of vertebrates, the response to a chronic stressor can also be characterised by changes in the leukocyte profile (Davis et al. 2008). The increase in the number of circulating heterophil counts, decrease in the number of lymphocytes (Siegel 1980; Satterlee et al. 1989), and raising heterophil/lymphocyte (H/L) ratios provide a reliable and widely used assessment of stress in birds and reptiles (Gross and Siegel 1983; Maxwell 1993; Aguirre et al. 1995; Ots et al. 1998; Davis et al. 2008). The H/L ratio is known to increase in response to various stressors, including starvation, injuries, heat and psychological disturbance (Averbeck 1992; Vleck et al. 2000). Increasing evidence shows that variation of leukocyte counts can

predict bird survival during the breeding season (Hörak et al. 1999; Lobato et al. 2005; Hylton et al. 2006; Kilgas et al. 2006). However, less is known about the changes of leukocyte counts of wintering birds under conditions of low ambient temperatures.

Energy stress is probably one of the main factors responsible for mortality of wintering birds. To enhance feeding efficiency and reduce the individual risk of predation, many birds live in social groups outside the breeding season (Ekman 1989). However, in dominance-structured groups, the competitive superiority of dominant individuals may add uncertainty to feeding opportunities and survival prospects of subordinate flock members, thus affecting the latter's body condition. For instance, subordinates are forced to carry extra reserves of subcutaneous fat as a buffer against periods of high energy demand or food shortage (Ekman and Lilliendahl 1993; Krams 2000), the gathering of which likely increases their stress level.

In this field study, we investigated the leukocyte profile and muscle score as condition indices of members of dominance-structured flocks of Great Tits (*Parus major*) under natural temperature regimes ranging from mild to extremely low ambient temperature occurring during sudden cold spells. The Great Tit is a small non-hoarding passerine that stores the acquired fat reserves internally in order to fuel thermoregulatory and other metabolic needs. Several studies have shown that in Great Tits males always dominate females and, within the sexes, adults dominate juveniles (Saitou 1979; De Laet 1985; Pöysä 1988; Sandell and Smith 1991; Gosler 1996; Verbeek et al. 1996; Krams 1998a, 2000; Verbeek et al. 1999). Since rank-related access to resources usually results in better winter survival rates among dominants (Ekman and Askenmo 1984; Krams et al. 2001, 2010; Koivula et al. 1995), we predicted a dominance rank-related increase in heterophil counts, decrease in lymphocyte counts, and increase the H/L ratios under conditions of extreme low ambient temperature. We expected higher H/L ratios in adult and, especially, juvenile female Great Tits under conditions of extremely low ambient temperature when the dominant individuals are most likely to take advantage of their superiority in access to resources.

Methods

We studied Great Tits wintering in the city of Daugavpils (55°52'N, 27°12'E) in south-eastern Latvia. The data were collected before a cold spell and during a cold spell in the winter of 2006–2007 when the ambient temperature dropped down to -30°C during just one night, and it remained cold for more than 20 days.

Two days before the weather forecast predicted the onset of a cold spell at the beginning of February 2007, we

captured 56 Great Tits (13 adult males, 14 first year males, 14 adult females and 15 first year females). During this period of mild weather, the average temperature in the night was within the range of 5 to -3°C while the mean daytime temperatures varied from 8 to -1°C . The average temperature in the night during the cold spell was within the range of -28 to -32°C , with mean daytime temperatures from -18 to -24°C . On day 7 after the onset of the cold spell, we captured birds again (57 individuals, including 13 adult males, 14 first males, 15 adult females and 15 first year females). The third capture was carried out on day 14 after the onset of the cold spell when we captured 58 Great Tits (14 adult males, 14 first year males, 15 adult females and 15 first year females). Altogether we captured 171 Great Tits, never using the same individuals during successive captures for the evaluation of the body condition. Birds were caught using with mist nets located at permanent feeders provided with ad libitum sunflower seeds between 0900 and 1230 hours. We sexed and aged each bird (first year or adult), and took basic morphological measurements of tarsus length, muscle score, and body weight. Muscle score was assessed by visually inspecting the pectoral muscle in a scale of 0–3 according to Bairlein (1995). We punctured the tarsal vein of each individual and collected blood using a standard microhaematocrit capillary tube within 1 min of handling, and released the birds within 5–6 min of capture. All animal manipulations were carried out in accordance with the legal and ethical standards of the Republic of Latvia.

In order to determine the differential white blood cell count, we used approximately 15 μl of blood to make whole blood air-dried smears using the standard two-slide wedge procedure (Houwen 2002). The samples were fixed in methanol and later stained with Wright-Giemsa Quik stain. All smears were examined to obtain counts of lymphocytes and heterophils, which were identified according to the criteria of Hawkey and Dennett (1989). No fewer than 100 leukocytes were counted per slide, while keeping track of the number of fields of view. Only the areas of the slides with adequate numbers of red blood cells were examined, and the number of red blood cells in each field of view was estimated. From these data, we calculated the total number of leukocytes per 10,000 erythrocytes, as well as the proportion of each leukocyte type. To calculate lymphocyte and heterophil numbers per 10,000 erythrocytes, the proportions were multiplied by the total number of leukocytes per 10,000 erythrocytes.

We performed a Kolmogorov–Smirnov analysis to test the normality of haematological parameters as well as muscle score distribution, which, despite the fact that ratios such as the H/L index and counts of leukocytes often have non-normal distributions, indicated that our data were normally distributed. Although it has recently been shown

that the results of the GLM models containing interactions between categorical and continuous predictors may not be correct (Littell et al. 2002), we included the categorical mean daytime temperature in our models, since the ambient temperatures over the course of a day were found to be nearly constant during the cold spell. All tests were two-tailed and were performed using SPSS software (SPSS, Chicago, IL, USA).

Results

We found a significant heterogeneity in heterophil counts of wintering Great Tits (one-way ANOVA, $F_{3,171} = 49.44$, $P < 0.001$; Table 1). Both adult and first year males had less heterophils than adult and first year females (Tukey's test, $P < 0.01$; Table 2), while we did not find significant differences neither between adult males and first year males ($P > 0.05$), nor between adult females and first year females ($P > 0.05$) (Tables 1 and 2). These differences were consistent over the whole study period, which indicates the absence of main effect of time capture, ambient temperature, and their interactions (Table 2).

Wintering Great Tits differed significantly in their lymphocyte counts (one-way ANOVA, $F_{3,171} = 13.15$, $P < 0.001$; Table 1). The main effects of sex and time of capture on lymphocyte counts were significant while the effects of ambient temperature and age were not significant (Table 2). The sex \times time of capture interaction was significant, indicating that the decline in lymphocyte counts was significant in females (Tukey's test, $P < 0.001$). A significant interaction among the sex, time of capture and ambient temperature also indicated that increase of heterophil counts was significant only for females (Tukey's test, $P < 0.01$) under conditions of low ambient temperatures (Table 2). Lymphocyte counts of adult males and first year males remained the same over the whole study period and we did not find significant differences between them (Tukey's test, $P > 0.05$; Table 2).

The H/L ratio also differed among the members of the winter flocks (one-way ANOVA, $F_{3,171} = 13.15$, $P < 0.001$; Table 1). The main effects of sex and time of capture on the H/L ratios were significant while ambient temperature and age were found to be non-significant (Table 2). The sex \times time of capture interaction was significant, revealing that the observed increase in the H/L ratio was significant for females (Tukey's test, $P < 0.001$). A significant interaction between the sex and age revealed that the increase in the H/L ratio can be mainly attributed to the first year females (Tukey's test, $P < 0.001$). The sex \times ambient temperature \times age interaction was also significant, showing that the general stress level of first year females (Tukey's test, $P < 0.001$)

Table 1 Condition indices of wintering Great Tits (*Parus major*) under contrasting climate conditions

| Rank | Time of capture | Heterophils | | | Lymphocytes | | | H/L ratio | | | Muscle | | |
|--------------------|---------------------------|-------------|-------|----------|-------------|-------|----------|-----------|------|----------|--------|------|----------|
| | | Mean | SD | <i>n</i> | Mean | SD | <i>n</i> | Mean | SD | <i>n</i> | Mean | SD | <i>n</i> |
| Adult males | Before cold spell | 6.15 | 3.46 | 13 | 83.46 | 10.13 | 13 | 0.07 | 0.06 | 13 | 2.54 | 0.99 | 13 |
| | First week of cold spell | 5.46 | 2.73 | 13 | 83.08 | 8.43 | 13 | 0.07 | 0.03 | 13 | 2.46 | 0.78 | 13 |
| | Second week of cold spell | 5.43 | 2.93 | 14 | 74.14 | 16.55 | 14 | 0.07 | 0.06 | 14 | 2.29 | 0.73 | 14 |
| First year males | Before cold spell | 5.57 | 3.74 | 14 | 82.07 | 14.13 | 14 | 0.07 | 0.04 | 14 | 2.43 | 0.65 | 14 |
| | First week of cold spell | 6.21 | 4.12 | 14 | 78.50 | 13.43 | 14 | 0.08 | 0.05 | 14 | 2.36 | 0.63 | 14 |
| | Second week of cold spell | 5.71 | 4.48 | 14 | 71.5 | 18.74 | 14 | 0.08 | 0.09 | 14 | 2.21 | 0.70 | 14 |
| Adult females | Before cold spell | 10.14 | 6.10 | 14 | 77.07 | 10.28 | 14 | 0.13 | 0.10 | 14 | 2.00 | 0.60 | 14 |
| | First week of cold spell | 26.80 | 11.88 | 15 | 68.47 | 11.17 | 15 | 0.40 | 0.19 | 15 | 1.73 | 0.15 | 15 |
| | Second week of cold spell | 28.40 | 12.82 | 15 | 43.47 | 12.19 | 15 | 0.65 | 0.28 | 15 | 1.07 | 0.70 | 15 |
| First year females | Before cold spell | 11.53 | 5.05 | 15 | 78.80 | 10.11 | 15 | 0.15 | 0.08 | 15 | 1.8 | 0.80 | 15 |
| | First week of cold spell | 32.13 | 9.75 | 15 | 70.73 | 10.76 | 15 | 0.45 | 0.16 | 15 | 1.27 | 0.70 | 15 |
| | Second week of cold spell | 32.13 | 8.68 | 15 | 39.47 | 8.93 | 15 | 0.81 | 0.17 | 15 | 0.53 | 0.64 | 15 |

Table 2 The effects of sex (S), age (A), time of capture (TC), ambient temperature (AT) on the condition indices of wintering Great Tits (*Parus major*) (GLM analysis)

| | Heterophils | | | Lymphocytes | | | H/L ratio | | | Muscle score | | |
|-------------|-------------|----------|----------|-------------|----------|----------|-----------|----------|----------|--------------|----------|----------|
| | <i>df</i> | <i>F</i> | <i>P</i> | <i>df</i> | <i>F</i> | <i>P</i> | <i>df</i> | <i>F</i> | <i>P</i> | <i>df</i> | <i>F</i> | <i>P</i> |
| S | 1 | 201.86 | 0.001 | 1 | 35.76 | 0.001 | 1 | 228.52 | 0.001 | 1 | 61.02 | 0.001 |
| A | 1 | 1.01 | 0.32 | 1 | 0.004 | 0.95 | 1 | 0.80 | 0.37 | 1 | 5.57 | 0.02 |
| TC | 1 | 0.39 | 0.53 | 1 | 15.44 | 0.001 | 1 | 11.79 | 0.01 | 1 | 3.29 | 0.07 |
| AT | 3 | 0.65 | 0.58 | 3 | 0.99 | 0.40 | 3 | 1.07 | 0.36 | 3 | 0.05 | 0.98 |
| S × A | 1 | 2.35 | 0.13 | 1 | 0.17 | 0.68 | 1 | 4.02 | 0.047 | 1 | 4.09 | 0.045 |
| S × TC | 1 | 0.26 | 0.61 | 1 | 17.47 | 0.001 | 1 | 19.61 | 0.001 | 1 | 0.17 | 0.68 |
| S × AT | 3 | 0.95 | 0.42 | 3 | 2.42 | 0.07 | 3 | 1.30 | 0.28 | 3 | 0.96 | 0.42 |
| TC × AT | 1 | 3.21 | 0.075 | 1 | 0.09 | 0.76 | 1 | 1.11 | 0.29 | 1 | 0.26 | 0.61 |
| S × TC × AT | 1 | 0.28 | 0.60 | 1 | 5.35 | 0.02 | 1 | 0.87 | 0.35 | 1 | 0.05 | 0.83 |
| S × TC × A | 1 | 3.03 | 0.08 | 1 | 1.02 | 0.32 | 1 | 1.72 | 0.19 | 1 | 0.15 | 0.70 |
| S × AT × A | 3 | 2.24 | 0.09 | 3 | 0.35 | 0.80 | 3 | 4.32 | 0.006 | 3 | 2.31 | 0.08 |

increased under extremely cold ambient temperatures (Table 2).

There was a significant heterogeneity in the muscle score among individual Great Tits (one-way ANOVA, $F_{3,171} = 27.04$, $P < 0.001$; Table 1). The main effects of sex, time of capture and age were significant (Table 2). The sex × age interaction was significant (Table 2) indicating that the body condition of the first year females was worse than the body condition of other flock members (Tukey's test, $P < 0.001$).

Since it is not known whether the changes in muscle score affects haematological condition indices, we also evaluated the general relationship between muscle score and the heterophil and lymphocyte counts as well as the H/L ratios. We found a significant negative relationship between muscle score and heterophil counts ($r_s = -0.48$,

$P < 0.001$), and muscle score and the H/L ratios ($r_s = -0.54$, $P < 0.001$), while the relationship between the muscle score and lymphocyte counts was significantly positive ($r_s = 0.41$, $P < 0.001$).

Discussion

The leukocyte concentrations and H/L ratios of female Great Tits changed significantly as soon as the climate became colder. Heterophils are the primary phagocytic leukocytes that increase in numbers in response to stress (Jain 1986; Campbell 1995; Rupley 1997; Harmon 1998; Thrall 2004), whereas the H/L ratio is shown to be positively related to the magnitude of the stressor and to the circulating glucocorticoids (e.g. Davis et al. 2008).

Although the elevated heterophil counts of female Great Tits coincided with a significant decrease of the average ambient temperature from 0 to -32°C , the GLM did not reveal that the observed change in heterophil counts in female Great Tits was affected by cold. However, GLM analysis of the lymphocyte concentration and H/L ratios of females showed a significant change between the two contrasting regimes of ambient temperature. While the H/L ratio in females, especially in the first year birds, increased under conditions of the extremely low ambient temperatures, the leukocyte concentrations and the H/L ratio of males did not change during the whole period of cold weather. These results suggest that the differences in haematological parameters may arise as a result of the individual dominance rank within the wintering flocks of Great Tits. In high latitudes, the availability of food and roosting sites is considered to be key factors affecting winter survival (Ekman 1987; Koivula et al. 1995). The existing evidence shows that the probability of starvation increases under harsh climate conditions and that starvation risk may be dependent on intraspecific and interspecific competition that causes sex-related access to the contested resources (Ekman 1987; Krams 1998b; Krams et al. 2001). Agonistic encounters initiated by dominant individuals restrict the access of subordinate group members to food resources and roosting sites (Ekman 1987) which leads to an uncertainty of survival prospects and higher general stress levels. The high stress levels may be especially important during cold spells when starvation risk and energy expenditures while roosting suddenly increase due to dramatically decreasing ambient temperature (Krams et al. 2010).

An additional explanation for the elevated stress as evidenced by the higher concentration of heterophils in female individuals under the conditions of low ambient temperature concerns the energetic consequences of their body size (Calder 1974). When the ambient temperature decreases, metabolism in smaller individuals increases proportionally more compared to larger individuals, suggesting that smaller individuals need more energy and time to build larger body reserves before roosting. Since the social position of females interferes with their high metabolic requirements (Broggi et al. 2004), the disadvantage of small size may increase the stress level of these individuals.

The impact of raised stress levels are manifest not only in the increase in heterophilia but also in the decrease in lymphocyte numbers. In homeotherms, thermoregulation during the winter season is an energy-demanding process and therefore may constraint energy allocation to immune function. Hence, it is usually assumed that the energy-demanding immune responses are suppressed during the winter season when resources may have to be reallocated to other expensive functions such as thermoregulation, and

some previous evidence shows that cold stress depresses cellular immunity in animals (Regnier and Kelley 1981). In response to stressful conditions, lymphocytes adhere to the endothelial cells of blood vessels, and subsequently undergo transmigration from circulation into other tissues (Cohen 1972; Fauci 1975; Dhabhar 2002) which causes a significant reduction in their circulating numbers. Despite this, we did not observe lymphopenia in the birds 1 week after the onset of the cold spell. This is in concordance with other studies that have shown that thermal application of cold exposure does not cause a strong enough cold stress to induce a trade-off between the immune function and physiological adaptations to increased energy requirements in wintering individuals. Previous studies have also shown that a longer duration of cold stress generally enhances lymphocyte-mediated responses (Hangalapura et al. 2004), and that resource reallocation in response to an environmental challenge may not be immediate (Cichon et al. 2002; Ksiazek et al. 2003). Since we did not find an increase in heterophil counts after the cold spell in males, the lack of effects of cold stress may be the case for dominant individuals. However, the lack of change in lymphocyte counts in the blood of females 1 week after the onset of cold spell does not necessarily signify the lack of lymphocyte demand, but may result from an increased demand coupled with a more intense production of lymphocytes by immunocompetent organs in response to cold and social stress. In turn, the significant lymphopenia found in female Great Tits birds 1 week later (2 weeks after the onset of cold spell) could signal an immune suppression. This assumption is supported by a significant decrease of health condition of females, especially the first year individuals, as evidenced by a decline in their muscle scores which were not observed in male Great Tits. The shift of the principal immunological mechanism away from lymphocyte-mediated responses, which have high metabolic demands due to rapid cell proliferation, may help females to conserve nutrients (Tschirren and Richner 2006). Because the less expensive immune mechanisms do not retain immunological memory and are less specific as lymphocyte-mediated mechanisms, a modest decrease in immunocompetence is expected in individuals having restricted access to the contested resources. Although immunosuppression incurs costs, the benefits of surviving the cold spell makes immunosuppression a beneficial tactic in terms of long-term fitness provided that innate immunity is not excessively activated which may be very expensive in terms of energy and immunopathology (Kim et al. 2007). However, during times of higher energy demand baseline levels of protection may also be maintained by other immune system components in circulation such as complement, natural antibody and mannan-binding protein providing protection against pathogens (Dhabhar and

McEwen 1999; Lee 2006; Buehler et al. 2008, 2009; Martin et al. 2008; Martin 2009), which should be considered for the future studies on bird responses to extremely low ambient temperatures.

Previous experimental evidence shows that increased thermoregulation during cold spells may incur substantial costs on immunity. The immune response of mice against sheep red blood cells exposed to long-lasting cold stress was found to be significantly lower than in mice exposed to brief cold stress (Cichon et al. 2002). The duration of the cold spell in our study supposedly was not long enough to cause cold-related stress in male birds that enjoyed the benefits of priority access to food. However, it is also possible that the effects of cold stress in our study birds were real but we failed to discriminate between the types of stress caused by cold versus position in social hierarchy. This calls for more research on the interplay between environmental stress, sociality, and immunity, in order for us to be able to quantify the exact costs and benefits of dominance rank and immunosuppression under harsh climate conditions.

It has been shown that migratory behaviour of birds exhibits great intraspecific variation. In partial migrant species, such as Great Tits, some birds migrate while the rest of the population remains sedentary (Lack 1944; Gautreaux 1980). In this species, females and juveniles normally constitute most of the migrants (Gautreaux 1980). Since we found that body condition of adult and especially first year females may be impaired due to position in dominance hierarchy, our results support both the ‘body-size hypothesis’ (Ketterson and Nolan 1976) and the ‘dominance hypothesis’ (Kalela 1954; Gautreaux 1980; Sutherland and Parker 1985) for the reasons behind differential migration. Both hypotheses predict that more females than males should migrate in species where males are larger than and dominant over females. Our results suggest that these patterns may derive from the fact that migration may be less costly for females in comparison to spending winter in higher latitudes where their survival is challenged by extreme environmental change.

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