

Changes in the corticosterone level in tooting male black grouse (*Tetrao tetrix*) infected with *Eimeria* spp.

R. Sokół and S. Koziątek-Sadłowska¹

Department of Parasitology and Invasive Diseases, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, 10-719 Olsztyn, Poland

ABSTRACT The black grouse (*Tetrao tetrix*) is a forest bird species critically endangered of extinction. Enclosed aviary breeding is among the measures taken to protect the species. Complex factors can affect its reproduction success, including coccidiosis. In this article, corticosterone level (as a reliable biomarker of stress in birds) and prevalence of *Eimeria* spp. were determined in male black grouse kept in aviary breeding center before, during, and after the reproductive season (called tooting). The correlation between those parameters was also analyzed. The corticosterone level was measured with noninvasive method in fecal samples in an immunoassay. The prevalence of *Eimeria* spp. was determined by the qualitative (Fülleborn's flotation) and quantitative method (McMaster's method as modified by Raynaud). Results show the occurrence of 3 species of *Eimeria* spp. in males: *Eimeria lyruri*, *Eimeria nadsoni*, and *Eimeria nonbrumpti*. Infestation with *E. lyruri* was

chronic in nature. A co-invasion of *E. lyruri*, *E. nadsoni*, and *E. nonbrumpti* was observed during (prevalence 60%) and after the tooting (prevalence 40%). The study showed no statistical changes in the oocysts per 1 g of feces (**CORTI**) and oocysts per 1 g of feces (**OPG**) in assays I-III and absence of correlation between CORTI and OPG. The results of this experiment do not support the hypothesis that chronic infestation with *Eimeria* spp. can induce chronic stress in grouse. But may suggest that males of black grouse are susceptible to infestation with other species of *Eimeria* spp. during mating season. The knowledge of the level of individual stress and parasitic infestation can be used to take protective actions for this bird species, especially to achieve higher survival rate and bird reproduction rate. Tangible effects will include an assessment of the relationship between individual susceptibility and chronic environmental stress caused by coccidia.

Key words: black grouse, corticosterone level, *Eimeria* spp., endangered species, stress

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INTRODUCTION

Corticosterone is the main stress hormone of the glucocorticosteroid group. (Campo et al., 2008). It is secreted after activation of the hypothalamic–pituitary–adrenal axis with the participation of environmental stressors, which include such factors as limited amount and/or quality of feed, variable environmental conditions, and predator pressure. Because the degree of adrenal activation and the level of stress hormones released after stimulation of the gland is correlated with the condition of the organism, corticosterone concentration is a reliable biomarker of individual

adaptation (Möstl and Palme, 2002; Lèche et al., 2013; Alm et al., 2014). As a rule, corticosterone level is determined in the blood. The method has some limitations which result from an increase in stress level resulting from manipulations during the blood sample collection. Moreover, corticosterone secretion and its concentration in blood varies from one time of day to another (Touma and Palme, 2005). Its determination in droppings/feces, which has been applied in this study, is an alternative method (Alm et al., 2014). The hormone concentration in this medium is more stable and does not undergo large fluctuations, and sample taking is completely noninvasive (Sheriff et al., 2010; Parnell et al., 2015). This method has been successfully applied in capercaillies (Książek et al., 2017) and ruminants living in the wild (Korzekwa et al., 2017).

Eimeria spp. are parasitic protozoans mainly inhabiting the intestinal epithelium in animals. Individual coccidia species differ by oocyst morphology, time of sporulation, location in the host body (different

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¹Corresponding author: sylwia.koziatek@uwm.edu.pl

alimentary tract sections), and pathogenicity. Infestation takes place *per os* and results from ingesting invasive oocysts. Infestation results in epithelium damage in the alimentary tract, mainly in the intestines, which is accompanied by disorders of nutrient absorption, digestive enzyme secretion, peristalsis, and the intestinal microflora composition. Mass breeding creates favorable conditions for infestation in domestic fowl, and young birds are particularly susceptible to it. Globally, the economic loss caused by coccidia is estimated at approx. 1 billion USD, with the treatment costs accounting for approx. 30% of the amount (Danforth and Augustine, 1990). The disease caused by the parasite is called coccidiosis. The course of the disease can be acute, chronic, or subclinical. The latter causes barely perceptible physiological and pathomorphological changes (Sokół et al., 2014).

Black grouse (*Tetrao tetrix*) is a forest bird of the order Galliformes, family Tetraonidae. It inhabits the boreal zone and the mountainous areas of Eurasia, areas of the initial phase of forest succession of a low degree of afforestation and humid and marshy terrain. The grouse population in Europe has decreased since the 20th century. Currently, it is an endangered species and is under strict legal protection in many European countries (Głowaciński, 2001; Storch, 2007). Predator pressure and human activities, such as unsustainable forest management, intense tourism, and recreation, as well as global climate change, are among the main factors which have resulted in the population decrease. Enclosed breeding is among the measures taken to protect the species. This is extremely difficult, and only a small fraction of birds can be re-introduced (Rzońca, 2018). Additionally, the re-introduced species often fall prey to predators. It is estimated that only 20% of the birds survive the first year of life (Merta et al., 2009, 2013; Kobielski and Merta, 2018). Parasitic infestations are also an important factor which may have a negative effect on populations of forest Galliformes birds (Sokół and Gałęcki, 2018). Parasitic diseases have a negative impact on the host's general health status and may be fatal in severe cases. They also affect the color of feces and behavior, which makes a bird an easy prey for predators (Hudson et al., 1992; Dobson and Hudson, 1995). This study is a continuation of the research conducted in an aviary breeding center, in which grouse parasitic fauna was shown to be dominated by protozoans of the *Eimeria* genus. They accounted for 41% of all the parasites found in the feces (OPG 450-110000), and the infestation was chronic (Sokół and Gałęcki, 2018). The dominance of this parasite in forest Galliformes birds (black grouse and capercaillie) has also been noted by Obeso et al., (2000); Millán et al., (2008) and Jankovska et al., (2012).

The following 2 theses have been put forth based on these findings. According to the first thesis, chronic infestation with coccidia results in chronic stress in black grouse. According to the second thesis, since the tooting, male bird is also accompanied by increased stress, it can increase the susceptibility to infestation with *Eimeria*

spp. Therefore, the aim of this study was to determine the level of stress by determining the corticosterone level in the feces to determine the prevalence of *Eimeria* spp. by the qualitative (Fülleborn's flotation) and quantitative method (McMaster's method as modified by Raynaud), as well as to demonstrate the correlation of both indices in the male grouse before, during, and after the reproductive season (tooting).

MATERIALS AND METHODS

Birds and Fecal Sample Collection

The experiment was conducted on 5 male grouse aged 2 to 3 yr, intended for breeding, kept at the grouse breeding center in enclosed aviary in the north of Poland (Spychow Forest District). The birds were fed with feed mixes intended for pigeons, with the diet being supplemented with dried cranberries and cowberries. Water was supplied *ad libitum* in containers placed in the aviaries once daily. Samples of fresh feces, approx. 10 g each, collected in the aviaries with a grate, individually for each bird, were used as the study material. The samples were collected 3 times: before reproductive season started—early March (assay I), in the peak of reproductive season—late April (assay II), and after reproductive season ended—late May (assay III).

Statement on the Welfare of Animals

The study involved no procedures on live birds. A noninvasive method of corticosterone and parasite determination in feces excreted to the environment was applied.

Analysis of Corticosterone Concentration/Level

The level of corticosterone (further referred to as CORTI) was determined in an immunoassay with a Corticosterone ELISA Kit (item no. 501320, Cayman Chemical, Ann Arbor, MI). Samples were purified and proceed according to the manufacturer's procedure. The linearity of dilution was determined before the assay (Figure 1), and the recovery rate was calculated (Table 1) by comparing the observed and expected values. To this end, a series of 3 dilutions were tested (1:10, 1:20, 1:30) for 2 independent samples. The procedure determines the predictability of a natural sample recovery for a known dilution in the standard range. The recovery rate from comparisons between serial samples of diluted feces samples and the values for the calibration curve was summed up in Table 1. Based on the results, an analysis was performed of samples in the 1:10 dilution, each in 2 replicates. The optical density of each sample was determined using an automated microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) equipped with a 405-nm wavelength filter.

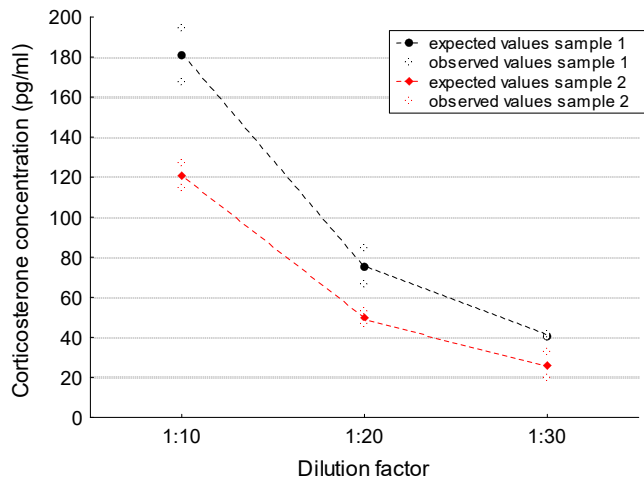


Figure 1. Expected and observed values obtained from the linearity of dilution test. The linearity of dilution was assessed by analyzing (in duplicate) serial dilutions (1:10, 1:20 and 1:30) of two independent samples and then calculating the rate of recovery by comparison between the observed and the expected values. This procedure assesses the predictability of natural sample recovery for a known dilution of a standard range. The rates of recovery from the comparisons between serial diluted feces extract samples and standard curve values are summarized in Table 1.

Parasitological Examination

The feces samples were examined for the presence of *Eimeria* spp. oocysts using the Fülleborn flotation method with Darling's solution (saturated aqueous solution of NaCl/glycerol, 1:1) (Gundlach and Sadzikowski, 2004). Subsequently, McMaster's quantitative method, as modified by Raynaud (Raynaud, 1970), was applied to determine the number of oocysts per 1 g of feces (OPG). The preparations were viewed under an optic microscope (Olympus) with 400 \times and 1000 \times magnification. Parasites were identified as *Eimeria* spp. based on morphological and morphometric features of the oocysts found, using the Olympus image analysis software and a parasitological atlas (Hendrix and Robinson, 2016).

Statistical Analysis

The statistical significance of changes of CORTI and OPG in different study periods (assays I-III) were analyzed with the Friedman's test ($P < 0.05$). The relationship between CORTI and OPG was examined by Spearman's R correlation. The parameters were considered under study individually. The analyses were performed with Statistica 13.1 software under Windows.

RESULTS

The level of corticosterone, number of oocysts per gram of feces, and the percentage share of different *Eimeria* species in male grouse before (assay I), during (assay II), and after (assay III) reproductive season is presented in Table 2. A median number for the CORTI in assays I-III was 272,463; 217,143; and 204,342, respectively. The

Table 1. Percentage of recovery calculated from comparisons between the expected and observed values within 3 applied dilutions of 2 independent samples run in duplicate.

Sample	Dilution factor	Testosterone pg/ml	Rate of recovery %
1	1:10	180.709	
		167.239	92
		194.180	107
	1:20	75.265	
		66.080	88
		84.449	112
1:30	40.646		
	39.930	98	
	41.361	102	
2	1:10	120.650	
		114.339	95
		126.961	105
	1:20	49.920	
		46.768	94
		53.072	106
	1:30	28.033	
		24.534	88
		31.532	112

The expected values for each dilution are shown in bold. A matrix effect is present when the recovery rate lies considerably below 80% or above 120%.

median value for the OPG in assays I-III was: 150; 1550; and 1000, respectively. A detailed parasitological examination revealed the presence of 3 *Eimeria* species: *Eimeria lyruri*, *Eimeria nadsoni*, and *Eimeria nonbrumpti*. *E. lyruri* was the dominant species throughout the study. Its following prevalence values were determined in assays I-III: 100, 80, and 100, and the percentage share of oocysts was 98, 86, and 90. It was nearly the only species found in assay I (*E. nadsoni* was found in 1 bird, accounting for 10% of all oocysts). Co-invasion of *E. lyruri*, *E. nadsoni*, and *E. nonbrumpti* (prevalence of 60%) was found in assay II; only *E. lyruri* was found in 2 birds. Co-invasion of these 3 species occurred in 2 birds in assay III (prevalence 40%), and co-invasion of *E. lyruri* and *E. nadsoni* was found in 1 bird (prevalence 20%). The mean percentage of oocyst of each species: *E. nadsoni* and *E. nonbrumpti* in the examinations was 10%.

Friedman's test ($P < 0.05$) showed no statistically significant changes of the CORTI (chi-square ANOVA [$n = 5$, $df = 2$] = 1.6; P -value = 0,45) and OPG (chi-square ANOVA [$n = 5$, $df = 2$] = 1.6; P -value = 0,45) in the study periods (assays I-III). Spearman's R correlation analysis showed absence of the correlation between CORTI and OPG ($R = 0,16$; $P < 0,05$).

DISCUSSION

Cascade hormone secretion is a physiological response to a stressor. First, within a few seconds of exposure, catecholamines are secreted, which accelerate the heart and breathing rate, increase the blood pressure, slow down the alimentary tract motor activity, and glucose supply release to blood. Subsequently, approximately 1 h after exposure to a stressor, glucocorticosteroids are secreted. Their secretion profile is species-dependent (it is corticosterone in birds). These hormones increase the glucose

Table 2. Corticosterone level, number of oocysts of *Eimeria* spp. in feces samples depending on the time of examination.

Assay	Sample/male no.	Corticosterone level pg/ml	Number of oocysts/1 g (OPG)	Percentage share of different <i>Eimeria</i> species		
				<i>E. lyruri</i>	<i>E. nadsoni</i>	<i>E. nonbrumpti</i>
I	1	147.295	150	100	-	-
	2	181.605	78	100	-	-
	3	955.585	150	100	-	-
	4	278.257	78	100	-	-
	5	272.463	2100	90	10	-
II	1	144.230	1550	85	10	5
	2	166.478	0	-	-	-
	3	337.202	12,600	85	5	10
	4	217.143	2500	75	10	15
	5	718.313	150	100	-	-
III	1	272.065	388	100	-	-
	2	204.342	2500	80	10	10
	3	159.607	3570	90	10	-
	4	234.525	1000	80	10	10
	5	192.318	78	100	-	-

concentration in blood by inducing glucogenesis and the secretion of erythropoietin. Short-term stress (eustres) mobilizes the body to respond and adapt to new environmental conditions. The prolonged action of a stressor and/or simultaneous action of several stressors leads to chronic stress (distress), which results in the secretion of proinflammatory substances and immunosuppression. Birds exposed to prolonged stress are more susceptible to infections. They can also experience reproductive problems. It has been demonstrated that stress hormones can be secreted in states of strong excitement, which are not a threat, for example during copulation or birth (Selye, 1950; White and Porterfield, 2012). The stress hormone level in grouse was found to increase as a response to human presence (tourist activities) in winter (Thiel et al., 2011). It was demonstrated in other hormones, for example in willow ptarmigan, that an increase in testosterone levels in males increases the parasitic invasion intensity. It has been shown in studies by these authors that infestation with *E. lyruri* was chronic in nature. The changes in the CORTI and OPG in assays I-III were not statistically significant. The intensity of infestation (OPG) did not correlate with the CORTI. During and after the tooting, a co-invasion of *E. lyruri*, *E. nadsoni*, and *E. nonbrumpti* was observed (Table 2). The mating season starts in mid-March and lasts to the end of May. The highest activity of males is observed between 15 April and 15 May. During that time, males gather in large groups and emit characteristic sounds to lure females, spreading their tails like fans. (Höglund et al., 1992; Höglund and Alatalo, 1995). It is the period of high activity and contacts between birds, which may have contributed to an increase in susceptibility to infestation with *E. nadsoni* and *E. nonbrumpti* and an increase in infestation with *E. lyruri*.

Because the reference values of corticosterone level in grouse are not known (no literature data), the results of this experiment do not support the hypothesis that chronic infestation with *Eimeria* spp. can induce chronic stress. But may suggest that males of black grouse are susceptible to infestation with other species of *Eimeria*

spp. at moments of increased effort (e.g., mating calls). To confirm beyond doubt, the theses in consecutive studies, a range of reference values for corticosterone in grouse should be established, considering the birds' sex, and CORTI and the prevalence of parasitic infestations should be monitored in an annual cycle.

When it comes to the research approach, the literature data show that metabolites of corticosterone in feces appear at different times depending on the animal species—12 to 24 h within exposure to a stressor. It has been shown that it is approx. 1 to 3 hs in grouse (Thiel et al., 2005). Oocysts do not start appearing in feces until approx. 7 D after infiltrating the host's body through the alimentary route. They are excreted within a few weeks. Therefore, it can be assumed that the corticosterone level and OPG determination is reliable, and it illustrates the relationship under study.

The knowledge of the level of individual stress and parasitic infestation can be used to take protective actions for this bird species, especially to achieve higher survival rate and bird reproduction rate. Tangible effects will include an assessment of the relationship between individual susceptibility to chronic environmental stress caused by coccidia.

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