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THE EFFECT OF COCCIDIANS ON THE CONDITION AND IMMUNE PROFILE OF MOLTING HOUSE SPARROWS (*PASSER DOMESTICUS*)

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ABSTRACT.—Feather molt is recognized as an energetically costly activity for birds, and parasite infestation during molt has the potential to reduce host fitness because parasites sequester essential nutrients and stimulate the immune system. We manipulated the coccidian parasite load of captive male House Sparrows (*Passer domesticus*) by suppressing the natural infection of control birds with an anticoccidial drug and infecting experimental birds with coccidian oocysts. Following infestation, the effect of chronic coccidian infection on individual condition, molt and 12 indices of physiological and immunological function was assessed. We found a significant positive relationship between infestation and heterophil/lymphocyte ratio measured at capture, indicating infection-induced stress. We also found that coccidians negatively affected feather growth during molt: the increase in wing length of the non-infected birds was significantly higher than that of infected birds. In comparison to control birds, infected birds showed a significantly higher concentration of white blood cells and greater blood bactericidal activity. There was also a positive correlation between infection intensity, agglutination and lysis of blood in the experimentally infested birds, which indicated activation of the constitutive innate immune system during infection. Finally, the total antioxidant capacity of the blood increased significantly, while the carotenoid concentration decreased significantly in infected compared with control birds. Therefore, we showed that coccidians stimulated several measures of the constitutive innate immunity, including the bactericidal activity of the blood, and that coccidians can have significant negative effects on the health and possibly fitness of molting House Sparrows. *Received 4 June 2010, accepted 17 December 2010.*

Key words: aviary, bacteria killing capacity, complement, experimental infection, feather quality, immune response, *Isospora*, natural antibodies.

Efecto de los Coccidios sobre la Condición y el Perfil Inmunológico de *Passer domesticus* durante la Muda

RESUMEN.—Se reconoce que la muda de las plumas es una actividad energéticamente costosa para las aves y que la infestación de parásitos durante la muda tiene el potencial de reducir la adecuación biológica del hospedero debido a que los parásitos secuestran nutrientes esenciales y estimulan el sistema inmune. Manipulamos la carga parasitaria de coccidios de machos cautivos de *Passer domesticus* mediante la supresión de la infección natural de las aves control con una droga anti-coccidios e infectando experimentalmente algunas aves con oocitos de coccidios. Luego de la infestación, se evaluó el efecto de la infección crónica de coccidios sobre la condición individual, la muda y 12 índices de la función fisiológica e inmunológica. Encontramos una relación positiva significativa entre la infestación y el cociente heterófilos/linfocitos medido en el momento de captura, lo que indica estrés inducido por la infección. También encontramos que los coccidios afectaron negativamente el crecimiento de las plumas durante la muda: el aumento en la longitud del ala de las aves no infectadas fue significativamente mayor que el de las aves infectadas. En comparación con las aves control, las

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aves infectadas mostraron una concentración significativamente mayor de glóbulos blancos y una mayor actividad bactericida de la sangre. También hubo una correlación positiva entre la intensidad de la infección, la aglutinación y la lisis de la sangre en las aves infestadas experimentalmente, lo que indicó la activación del sistema inmune innato constitutivo durante la infección. Finalmente, la capacidad antioxidante total de la sangre incrementó significativamente, mientras que la concentración de carotenoides disminuyó significativamente en las aves infectadas en comparación con las aves control. Por lo tanto, demostramos que los coccidios estimularon varias medidas del sistema inmune constitutivo, incluyendo la actividad bactericida de la sangre, y que los coccidios pueden tener efectos significativos negativos en la salud y posiblemente en la adecuación biológica de los individuos de *P. domesticus* durante la muda.

Coccidians are microscopic, unicellular parasites that inhabit the epithelium of the intestine (Allen and Fetterer 2002). Among the most prevalent of avian parasites (Greiner 2008), coccidian infection can stimulate the immune system and, as is apparent from studies on poultry and several wild avian species, may negatively effect host fitness (e.g., Allen and Fetterer 2002, Hõrak et al. 2004, Greiner 2008, Mougeot et al. 2009). In wild birds, coccidian infestation can affect both the physical appearance, via a reduction in the signaling value of morphological traits (Hill and Brawner 1998, Hõrak et al. 2004, Baeta et al. 2008, Mougeot et al. 2009), and the behavior of an individual (Buchholz 2004, Aguilar et al. 2008, Dolnik and Hoi 2010). Furthermore, infestation can significantly reduce an individual's condition (Allen and Fetterer 2002, Hõrak et al. 2004, Aguilar et al. 2008, Mougeot et al. 2009), and in some situations, may lead to death (Gill and Paperna 2008, Greiner 2008, Krautwald-Junghanns et al. 2009).

On the basis of studies of poultry and captive Eurasian Kestrels (*Falco tinnunculus*), the major immune response to coccidian infection is the production of T-cells by the gut-associated lymphoid tissues (Allen and Fetterer 2002, Lemus et al. 2010). Furthermore, the activation of different leukocyte types (e.g., macrophages and natural killer cells) during coccidian infection suggests that aspects of innate immunity may play a role in the defense against coccidian infection. By contrast, antibody mediated humoral immune responses appear to play a minor role in combating coccidiosis (Allen and Fetterer 2002). An important consequence of immune system activation is an increase in the production of free radicals (reactive oxygen and nitrogen species, RONS), which can significantly alter an individual's resistance to, and immunity against, coccidians (Møller et al. 2000, Allen and Fetterer 2002). Carotenoids and other antioxidant biomolecules that contribute to the total antioxidant status (TAS) of the organism can scavenge free radicals produced during immune system activation and can thereby minimize potential oxidative damage and reduce autoreactivity and self-harm (Møller et al. 2000).

Typically, individuals infected with coccidians are asymptomatic. However, under conditions of stress (e.g., malnutrition, energetically and/or nutritionally demanding activities) the intensity of infestation may increase, leading to potential fitness costs for the infected host (Allen and Fetterer 2002, Greiner 2008). Molt is recognized as an energetically costly activity for birds (Lindström et al. 1993). During molt, stressors such as parasite infestation have the potential to compromise an individual's fitness because the conditions prevailing during plumage replacement determine the quality of the feathers that are produced, and ultimately, the flight performance and thermal insulation of individuals (Swaddle et al. 1996). Coccidians reduce general absorption, drain essential nutrients (e.g., amino acids), and stimulate the immune system (Allen and Fetterer 2002). Thus, coccidian infection during molt can induce a range of costs, including prolonged molt, reduced feather quality, and increased post-molt mortality resulting from insufficient insulation and reduced predator escape ability. Furthermore, as most passerines molt only once per year and feather quality cannot be enhanced between molts, these effects can be long-lasting. Despite the potential importance of coccidian infestations during the molt period, only a few studies have investigated the effects of parasite infestation during molt (Hill and Brawner 1998, Hill et al. 2005). And contrary to expectation, Pap et al. (2009) did not find any effect of coccidians on molt in a previous experimental study on the House Sparrow (*Passer domesticus*). In the present study, we extend the work of Pap et al. (2009) and simultaneously modify diet and coccidian infection to evaluate whether being fed a protein rich diet in a previous study masked the predicted negative effect of these parasites on molting House Sparrows.

Knowledge of how the immune system of an individual responds to parasites is the first step in understanding the role of immunocompetence in host–parasite evolution. Studies on wild birds have demonstrated that several physiological and immune measures respond to coccidian infestation (Hõrak et al. 2004, Mougeot et al. 2009, Pap et. al. 2009, Lemus et al. 2010). However, the mechanism of immune defense and the sensitivity of different immune measures to coccidians remain largely unexplored (Lemus et al. 2010). Based on studies of poultry and free-living birds, we hypothesize that humoral component of the immune response to coccidian infection would be secondary to the primary response provided by the cellular and innate immune branches of the immune system. Thus, the objectives of the present study are to (1) investigate the consequences of chronic infection by *Isospora* coccidians on molt, feather quality and condition in House Sparrows; and (2) describe the immunological and physiological processes that accompany coccidian infection in this species using a range of common measures of immunity and physiology. To accomplish our objectives, we infected experimental birds with coccidian oocysts (2 dosage levels) and treated control birds with an anticoccidial drug (2 dosage levels). We then assessed the process of molt, feather quality, and a number of physiological and immune responses of birds belonging to the different experimental groups.

Methods

General Procedure

Fifty-nine adult male House Sparrows were caught with mist nets (Ecotone, Poland) at two farms near Cluj Napoca, Romania (46°46′N, 23°33′E) during two capture sessions on 20 and 21 July 2009 (day 0; see Fig. 1). Birds were then transported to the campus of the Babeş-Bolyai University in Cluj Napoca and housed in four groups ($n = 14$ to 15 birds group^{-1}) in outdoor aviaries ($5 \times 2 \times 2.5$ m; length × width × height). Throughout the experiment, birds were fed *ad libitum* with a mixture of seeds containing ground

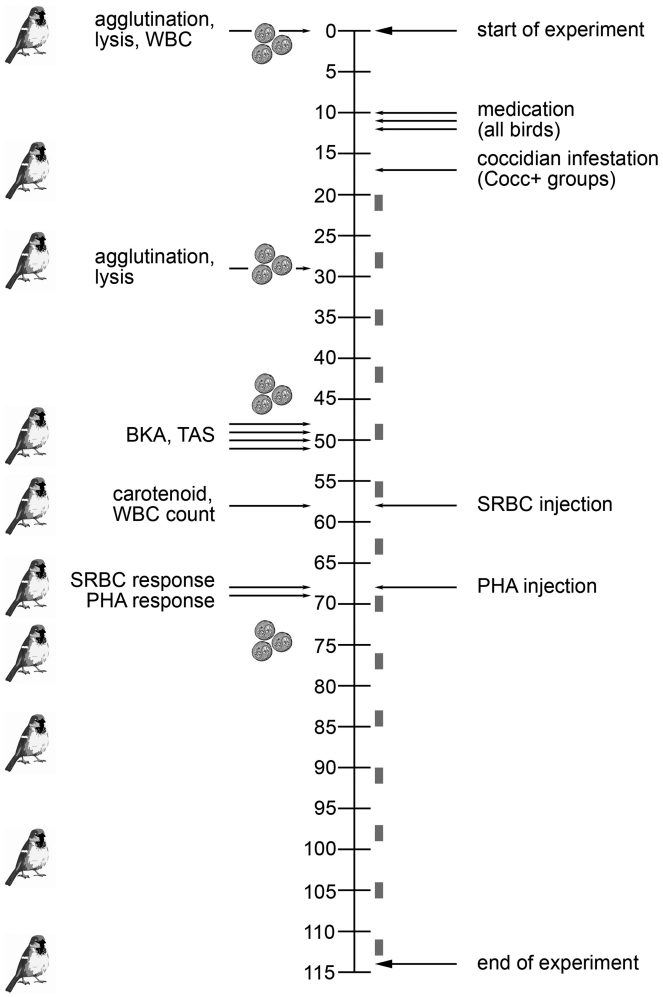

Fig. 1. Schematic representation of the experimental protocol used in this study (day 0 = 20 and 21 July, day 114 = 12 November). The narrow arrows to the right of the scale indicate days on which treatments were given (grey rectangles = coccidian medication in Cont groups; 2 days per week), whereas the narrow arrows to the left of the scale give the dates on which different measurement were made (sparrow insets = molt was scored and birds were weighed; oocyst drawings = the date of measures of infection). For details see Methods.

corn, sunflower, wheat and oat. In addition, on every second day birds were given a protein supplement that alternated between two grated boiled eggs or mealworms (two mealworms bird^{-1}). Thus, the protein content of the food was half of what we applied in a previous study on this species (Pap et al. 2009). Fresh tap water was provided daily. No birds had begun to molt prior to our experiment. Of the 59 captured House Sparrows, one individual died from unknown causes during the first week of captivity. All other individuals were released in good health at the end of the experiment.

Experimental Protocol

At capture, we measured wing length, body mass and tarsus length of all birds and prepared a blood smear for leukocyte count (see methods below). At the same time, we collected 75 µL of blood from the brachial vein in heparinized capillary tubes, separated the plasma from blood cells by centrifugation and stored these at –20°C until hemolysis–hemagglutination assay (see below). Following capture, birds were allowed to acclimatize until 23 July (day 2; Fig. 1). That day, birds were placed in individual outdoor cages in order to quantify the natural level of chronic coccidian infestation. To assess infestation, we measured the rate of oocyst-shedding (i.e., the number of oocysts g^{-1} of feces day^{-1}; see below) over a two day period (days 2–3). The next day (day 4), we randomly assigned birds to one of the four aviaries and assigned each aviary to one of four experimental groups: (1) medicated with low dose of anticoccidial drug (1 mL Baycox [25 mg toltrazuril] in 1 L drinking water) (Cont1; $n = 14$); (2) medicated with high dose of anticoccidial drug (2 mL Baycox [50 mg toltrazuril] in 1 L drinking water) (Cont2; $n = 14$); (3) infected with 2,000 coccidia oocysts (Cocc2000; $n = 15$); and (4) infected with 5,000 coccidia oocysts (Cocc5000; $n = 15$).

Treatments were applied as follows (see Fig. 1). On day 10, all birds were treated with an anticoccidial drug provided in their drinking water for three (10–12) days (Baycox, Bayer, 2.5% [25 mg toltrazuril in 1 mL]; we dissolved 1 mL Baycox in 1 L drinking water). Five days later (day 17) birds in the Cocc2000 and Cocc5000 groups were inoculated orally with *Isospora* oocysts diluted in physiological solution (see Pap et al. 2009), while birds from Cont1 and Cont2 groups received only the same volume of physiological solution. Because the effect of anticoccidial drug lasts only for a few days (Hõrak et al. 2004, Pap et al. 2009), after which oocysts reappear in the feces due to natural reinfection, we treated birds in Cont1 and Cont2 groups with low and high dosages, respectively, of anticoccidial drug weekly (2 days per week) from day 17 until the end of the experiment. In addition to maintaining reduced parasite load, this experimental approach allowed us to test the potential side effect of anticoccidial drug on bird condition, molt, and physiological and immunological measures. Eleven days after inoculation, oocyst shedding was measured for the second time by placing birds in individual cages for two days (days 28–29). At the same time (day 28), we collected 75 µL of blood from all birds for hemolysis–hemagglutination assay. The infestation level was assessed for the third time between days 44–45, followed by a blood collection (days 48–51) to assess the TAS and bacteria killing ability (*Escherichia coli* and *Staphylococcus aureus*) of birds. Next, on day 58, we collected blood from all the birds for carotenoid analyses and prepared a blood smear (see above) for leukocyte count. Birds were then injected with 100 µL 20% fresh sheep red blood cells (SRBCs) suspension into the pectoralis muscle (see Pap et al. 2008) in order to assess the humoral immune response against a novel antigen. In our previous studies on House Sparrows we had shown no detectable antiSRBC titer under natural conditions (Pap et al. 2008). Ten days after the SRBC injection (day 68), we collected blood samples to measure antibody titer against the SRBC. Following blood collection, we injected birds with phytohemagglutinin (PHA) solution in order to measure the inflammation immune response (Martin et al. 2006; Tella et al. 2008; Vinkler et al. 2010). Finally, coccidian infestation was measured once again during a two-day period (days 74–75) using the methods set out below.

On days that birds were handled (days 2, 17, 28, 48–51, 58, 68, 76, 87, 101, 114; see Fig. 1), we measured body mass and assessed molt of the primaries according to the following scheme: we scored the dropped feathers as 1, a quarter-, half- or three-quarter regrown

feathers as 2, 3 and 4, respectively, and the fully-regrown feathers as 5 (for details, see Pap et al. 2008). Old feathers received a score of 0. On each occasion, we calculated the molt score for each individual, where molt score is the sum of the scores of individual primary feathers (total 9, the tenth is rudimentary) and ranges from 0 (before the beginning of molt: all primaries are old) to 45 (molt finished: all primaries have been completely replaced).

At the end of molt (when all primary feathers were replaced) we measured wing length, and then we plucked primary 2 (P2) and P7 feathers from both wings to assess the effect of experimental manipulation on the change in wing length between pre-molt and post-molt and on the quality of the new feathers. We characterized the quality of feathers using three variables: feather length, rachis diameter and feather mass (see Dawson et al. 2000; Pap et al. 2008). Feather length was measured with a ruler to the nearest 0.5 mm and rachis diameter was measured with a digital caliper (0.01 mm accuracy) at 1.5 cm from the base of the shaft. Feathers were weighed on an electronic balance (10^{-4} g accuracy) and, for all three measures, the mean of P2 and P7 from the left and right wing were used.

In a previous study on the same House Sparrow population, we demonstrated that *Isospora lacazei* was the most common coccidian in the fecal samples (Pap et al. 2009). As coccidians from the genus *Isospora* shed oocysts predominantly during the late afternoon (Filipiak et al. 2009), feces were collected just before sunset. The number of oocysts was counted in McMaster chamber as described by Pap et al. (2009) and the concentration was expressed as number of oocysts g^{-1} of feces. The mean values of the oocysts' number collected during the two-days sessions were used in the analyses.

Immunological and Physiological Measures

Leukocyte count.—To count leukocytes, a drop of blood was smeared on a microscope slide, air-dried, fixed in Dia-Fix, and stained with Dia-Red and -Blue Panoptic (Diagon Ltd., Hungary). Smears were examined at 1,000× magnification and the proportion of different types of leukocytes was assessed by counting 50 leukocytes. The number of white blood cells of different types was expressed per approximately 10,000 erythrocytes. We excluded monocytes, eosinophils and basophils from the analyses because of their low concentration in the blood (less than 3 cells 10,000 erythrocytes^{-1}). Leukocytes were counted by the same person (GO), and were moderately to highly repeatable (heterophils: $r = 0.76$, df = 9 and 10, $F = 7.3$, $P < 0.01$; lymphocytes: $r = 0.66$, df = 9 and 10, $F = 4.9$, $P = 0.01$; total leukocytes: $r = 0.71$, df = 9 and 10, $F = 5.9$, $P < 0.01$; heterophil/lymphocyte (H/L) ratio: $r = 0.71$, df = 9 and 10, $F = 5.9$, $P < 0.01$).

Hemolysis–hemagglutination assay.—We assessed the constitutive humoral immunity (i.e., the levels of the natural antibodies [NAbs] and complement) using a modified hemolysis–hemagglutination assay (Matson et al. 2005; Pap et al. 2010). We described this method in detail elsewhere (Pap et al. 2010). Briefly, however, the only modification related to our previous work was that here we used freshly collected rat instead of rabbit red blood cell suspension (see Seto and Henderson 1968). In this assay, agglutination reflects the activity of the NAbs, while lysis represents the interaction between the NAbs and the complement (Matson et al. 2006; Buehler et al. 2008a).

Bacteria killing ability of the whole blood.—To reduce the negative effect of stress on immune responses (Buehler et al. 2008b), we collected 100 μL blood under sterile conditions from the brachial vein within 20 min after initial capture and divided this sample into two aliquots, one for the bacteria killing assay and one for TAS analysis (see below). We tested the bactericidal activity of the blood against standard laboratory strains of *E. coli* (ATCC# 10536) and *S. aureus* (ATCC# 6538P) following Pap et al. (2010).

Total antioxidant status.—TAS is an indication of the ability of the organism to defend itself against free radicals. TAS of plasma was determined using a commercial antioxidant kit (Antioxidant Assay kit, Cayman Chemical, Ann Arbor, Michigan, USA). Briefly, plasma was diluted 1:20 with assay buffer and the rest of the assay was performed using manufacturer's instructions.

Plasma carotenoids analyses.—Total carotenoids were extracted by treating 20 μL of plasma with 180 μL of absolute ethanol and the carotenoids concentration was determined by reading the absorbance at 450 nm with a BioTek Synergy HT microplate reader (BioTek Instruments, Winooski, VT, USA). Carotenoids concentration was calculated using a standard curve of pure lutein in the range of 1–25 μg mL^{-1} ($r = 0.999$) and reported as μg mL^{-1} (see Pap et al. 2009).

Measurements of antiSRBC titer.—Antibody titers were measured using a base-2 serial dilution hemagglutination test conducted with 15 μL of heat-inactivated plasma (30 min on 56°C) on U-shaped 96-well microtiter plates. Samples were serially diluted starting with 15 μL PBS, and to each well 15 μL of a 1% suspension of SRBC in PBS was added. Plates were incubated at 37°C for 1 hour. Titers are given as the $\log_2$ of the reciprocal of the highest dilution of plasma showing positive hemagglutination (Pap et al. 2008).

PHA-induced immune response.—Non-specific, inflammation immune response was measured using a common mitogenic challenge (PHA; L-8754, Sigma; e.g., Pap et al. 2009). We injected 100 μL of 1 mg mL^{-1} PHA solution in the left wing web. The immune cell activity was estimated by measuring the increase in thickness of the wing web 24 h after injection with a pressure sensitive spessimeter (accuracy 0.01 mm).

Statistical Analyses

The effects of experimental manipulation on coccidian infestation, body mass, duration of molt, and 12 physiological and immunological measures were analyzed using general linear models (GLMs), where the dependent variables were entered in separate models and the experimental groups were set as factors. Similarly, we tested the increase in body mass and molt score 11 days post infestation (dpi, between days 17 and 28), the change in total and differential leukocyte counts between days 0 and 58 and the change in agglutination and lysis between days 0 and 28. Birds originating from different farms were distributed randomly and evenly between the experimental groups. Furthermore, no difference was found between birds from the two farms in wing length ($F = 1.5$, df = 3 and 54, $P = 0.22$), tarsus length ($F = 0.8$, df = 3 and 54, $P = 0.51$), body mass ($F = 1.3$, df = 3 and 54, $P = 0.30$) and level of coccidian infestation ($F = 1.5$, df = 3 and 54, $P = 0.22$) at capture. Therefore, we did not control for farm identity in the statistical analyses. Sample sizes varied among analyses because some birds had missing or incomplete data (e.g., spoiled blood during centrifugation or low plasma

volume). Because of the skewed distribution of coccidians and leukocytes, we normalized them by $\log_{10}$-transformation and used transformed values in all subsequent analyses. Mean ± SD are shown throughout the text unless otherwise stated. All variables were considered significant at $P < 0.05$. All analyses were carried out in the STATISTICA, version 7.0, software package (StatSoft, Inc., Tulsa, Oklahoma).

RESULTS

Coccidian infestation and physiological measures at capture.—At capture all birds were infected by coccidians and the median number of oocysts g^{-1} feces was 162,137 (lower quartile: 86,829, upper quartile: 342,719, $n = 58$). Infestation level was independent of variation in wing length ($r = 0.06$, $n = 58$, $P = 0.64$), tarsus length ($r = -0.06$, $n = 58$, $P = 0.64$) and body mass ($r = -0.18$, $n = 58$, $P = 0.18$). H/L ratio was positively correlated with the number of oocysts shed in feces at the time of capture ($r = 0.27$, $n = 56$, $P = 0.04$; Fig. 2). In contrast, heterophil concentration ($r = 0.13$, $n = 56$, $P = 0.34$), lymphocyte concentration ($r = -0.03$, $n = 56$, $P = 0.85$) and total white blood cells (WBC) concentration ($r = 0.07$, $n = 56$, $P = 0.61$) were not associated with the intensity of infestation. Similarly, agglutination and lysis against rat red blood cells was not significantly associated with the level of infestation measured upon capture (agglutination: $r = -0.11$, $n = 52$, $P = 0.45$; lysis: $r = -0.17$, $n = 52$, $P = 0.24$).

The effect of treatment on coccidians, molt, feather quality and condition.—The anticoccidial drug and the experimental coccidian infestation significantly affected the number of oocysts shed in the feces (Fig. 3), as revealed by the significant group ($F = 56.3$, df = 3 and 54, $P < 0.0001$) and group × repeated measures interaction ($F = 12.4$, df = 9 and 54, $P < 0.0001$) in the repeated-measures GLM analysis, where the number of oocysts counted during the four sessions were entered as dependent variables and group was entered as a factor. On day 28 (11 dpi), Cocc and Cont groups differed significantly in oocyst count (planned comparison: $F = 5.4$, df = 1 and 54, $P < 0.05$). However, this difference was because of the higher number of oocysts in the Cocc2000 rather than in the Cocc5000 group ($F = 4.7$, df = 1 and 54, $P < 0.05$). On day 44, birds from both Cocc groups were significantly more infested than the Cont groups ($F = 62.7$, df = 1 and 54, $P < 0.0001$). No difference existed between the Cocc2000 and Cocc5000 ($F = 0.3$, df = 1 and 54, $P = 0.56$), or between the Cont1 and Cont2 groups ($F = 0.3$, df = 1 and 54, $P = 0.59$). The difference between experimentals and controls remained until the last coccidia sampling at day 74 (Cocc vs. Cont groups: $F = 112.6$, df = 1 and 54, $P < 0.0001$; Cocc2000 vs. Cocc 5000: $F = 0.2$, df = 1 and 54, $P = 0.64$; Cont1 vs. Cont2: $F = 3.6$, df = 1 and 54, $P = 0.07$).

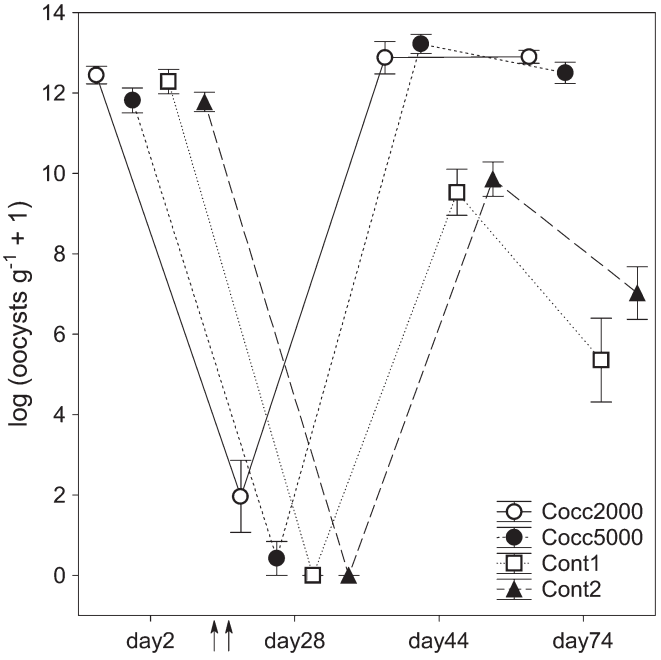

FIG. 3. Change in oocyst-shedding (mean ± SE) of House Sparrows in different groups during the course of the experiment. Individuals were put into the aviaries on 20 and 21 July (day 0). All birds were medicated for three days starting on day 10 (first arrow), followed by the reinfection of House Sparrows with 2,000 and 5,000 oocysts in the case of Cocc2000 and Cocc5000 groups, respectively, on day 17 (second arrow).

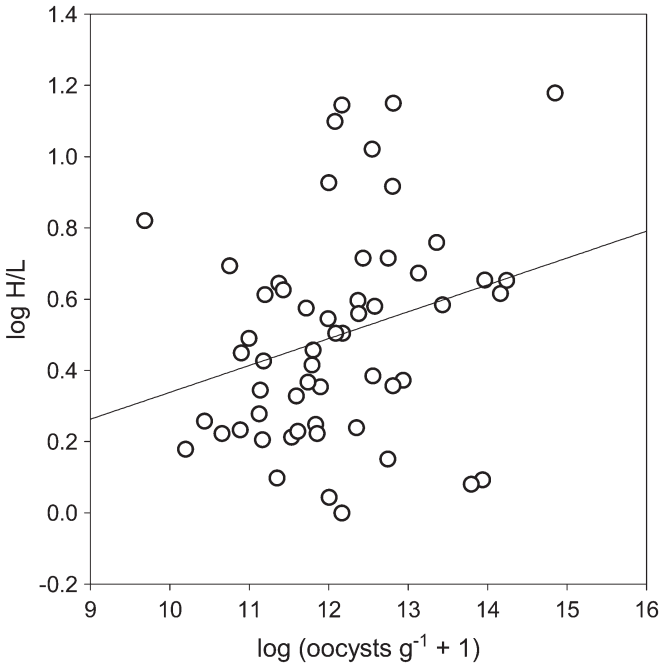

FIG. 2. The relationship between log H/L ratio and coccidian infestation of House Sparrows measured at day of capture and one day following the introduction of birds into the aviaries, respectively (all groups pooled).

Experimental manipulation had no effect on either the speed of molt (measured by the change in molting score between day 17 and day 28) or the duration of molt (i.e., the time taken for all primary feathers to grow following the loss of the first innermost primary feather; Table 1). Cont groups showed a significantly greater increase in wing length following molt compared to Cocc groups (Table 1, Fig. 4), whereas there were no significant effects of dosage (low vs. high dose anticoccidial drug, low vs. high infestation dose) on wing length. The mass of P7 was significantly

Table 1. Results of the general linear models that compared control and experimentally treated (coccidian-inoculated) House Sparrows for the absolute value and change of body mass, wing length, molting speed and on the size and mass of flight feathers. Groups that share letters (A or B) did not differ significantly based on Tukey *post hoc* test (see text).

Molt, body or plumage trait	Experimental groups (mean ± SD)				F[a]	P
	Cocc2000	Cocc5000	Cont1	Cont2		
Molt and body mass						
Molt duration (days)	74.5 ± 10.64	78.5 ± 10.43	77.2 ± 8.60	79.1 ± 9.98	0.6	0.62
Change in molting score (day 28 – day 17)	10.7 ± 2.60	9.9 ± 2.60	10.0 ± 2.32	9.4 ± 3.06	0.6	0.62
Change in wing length (post-molt – pre-molt)	0.53 ± 1.302^{A}	0.40 ± 1.242^{A}	2.14 ± 1.100^{B}	1.86 ± 1.350^{B}	7.4	0.0003
Change in body mass (day 28 – day 17)	2.1 ± 0.74	1.4 ± 0.51	1.5 ± 1.16	1.4 ± 0.86	2.1	0.11
Feather quality						
Primary 7 length (mm)	68.7 ± 1.60	68.1 ± 1.42	69.1 ± 1.50	69.1 ± 1.69	1.3	0.29
Primary 2 length (mm)	58.2 ± 1.48	57.2 ± 1.52	58.5 ± 1.24	58.2 ± 1.12	2.3	0.09
Primary 7 rachis width (mm)	1.08 ± 0.045	1.11 ± 0.040	1.09 ± 0.042	1.09 ± 0.024	1.5	0.24
Primary 2 rachis width (mm)	0.92 ± 0.044	0.93 ± 0.035	0.93 ± 0.033	0.92 ± 0.033	1.1	0.36
Primary 7 mass (1×10^{-4} g)	152.7 ± 9.82^{A}	149.5 ± 10.06^{A}	156.0 ± 5.52^{A,B}	162.5 ± 6.72^{B}	6.4	0.0009
Primary 2 mass (1×10^{-4} g)	103.2 ± 6.72	101.2 ± 4.74	103.9 ± 4.06	105.2 ± 4.51	1.5	0.22

[a]df = 3 and 54.

affected by coccidian infestation: birds from the Cocc groups grew lighter flight feathers than the Cont groups ($F = 13.8$, df = 1 and 54, $P < 0.001$; Table 1). Finally, experimental coccidian infestation had no effect on any other measure of feather quality or change in body mass (Table 1).

The effect of treatment on physiological and immune responses.—Birds experimentally infected with coccidia showed an increase in bacterial killing capacity of blood against *E. coli* and *S. aureus* (Table 2). On the other hand, the difference between Cocc5000 and Cocc2000 groups, and the difference between Cont1 and Cont2 groups were not significant. WBC, measured at day 58, differed significantly between experimental groups because total leukocyte count was significantly higher in the Cocc5000 compared with the Cont2 groups (Table 2). TAS increased significantly in Cocc5000 relative to Cont groups, whereas the concentration of plasma total carotenoids was significantly lower in the Cocc groups compared with the Cont2 group (Table 2). After controlling for the effect of experimental groups, TAS did not vary significantly with the level of circulating carotenoids ($F = 0.1$, df = 1 and 52, $P = 0.76$).

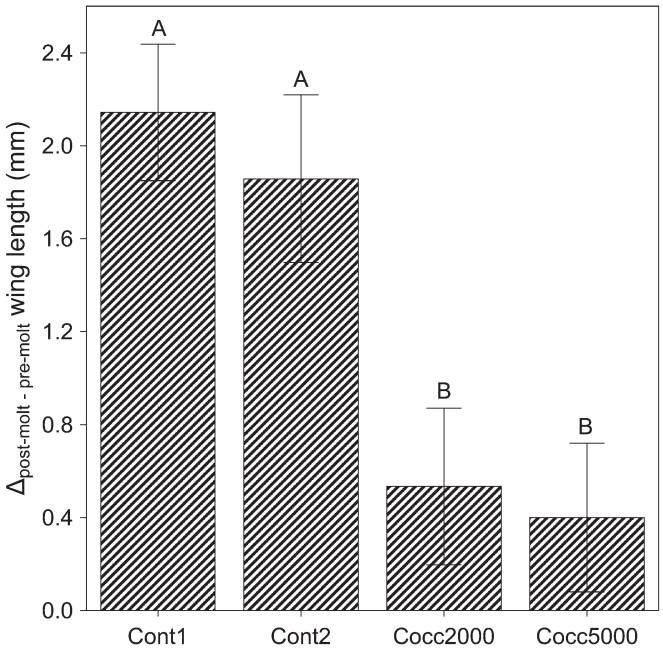

Fig. 4. Change of wing length in House Sparrows between length at capture and upon completion of molt (Δ, i.e., post-molt minus pre-molt value) between controls and birds that were experimentally inoculated with coccidians. Bars represent means ± SE. Groups that shared letters (A or B) did not differ significantly based on Tukey post hoc test (see text).

None of the other physiological or immunological variables were affected by the coccidian manipulation. Specifically, humoral immune response against SRBCs, the PHA swelling response, heterophil concentration, lymphocyte concentration and H/L ratio measured at day 58 (Table 2) and the change in leukocyte concentration (i.e., the difference between measurements taken at day 0 and 58) was similar between experimental groups (Δ log heterophil concentration: $F = 0.8$, df = 3 and 49, $P = 0.50$; Δ log lymphocyte concentration: $F = 0.8$, df = 3 and 49, $P = 0.51$; Δ log H/L: $F = 0.7$, df = 3 and 49, $P = 0.53$; Δ Log WBC: $F = 0.9$, df = 3 and 49, $P = 0.46$). Similarly, neither the change in agglutination nor lysis against rat red blood cells between days 0 and 28 differed between experimental groups (Δ agglutination: $F = 0.5$, df = 3 and 48, $P = 0.69$; Δ lysis: $F = 0.2$, df = 3 and 48, $P = 0.87$). Following infestation in the Cocc groups, agglutination and lysis was positively correlated with the infestation level measured at day 28 (agglutination: $r = 0.59$, $n = 30$, $P < 0.01$; lysis: $r = 0.62$, $n = 30$, $P < 0.0001$; Fig. 5.

Discussion

Coccidians can seriously affect the fitness of the wild birds (Greiner 2008) because they inhibit the uptake of dietary components from the alimentary tract and stimulate the immune system of the host (e.g., Hőrak et al. 2004, Baeta et al. 2008, Mougeot et al. 2009). In the present study, we did not detect any

Table 2. Results of the general linear models that compared immunological and physiological responses of House Sparrows treated as either controls or experimentals that were inoculated with coccidian parasites. Groups that share letters (A, B, or C) did not differ significantly based on Tukey post hoc test (see text).

	Experimental groups (mean ± SD)						
Responses	Cocc2000	Cocc5000	Cont1	Cont2	*F*	df	*P*
Log agglutination (day 28)	1.00 ± 0.745	0.52 ± 0.402	0.45 ± 0.724	0.56 ± 0.690	2.2	3 and 54	0.10
Log lysis (day 28)	0.57 ± 0.727	0.14 ± 0.287	0.22 ± 0.577	0.20 ± 0.424	2.0	3 and 54	0.12
E. coli killing capacity (days 48–51)	46.4 ± 19.52^{A}	41.7 ± 23.85$^{A, B}$	27.6 ± 15.53$^{B, C}$	22.7 ± 4.14^{C}	6.0	3 and 54	0.001
S. aureus killing capacity (days 48–51)	30.4 ± 14.06^{A}	27.6 ± 10.06$^{A, B}$	16.3 ± 8.75^{C}	18.2 ± 5.05$^{B, C}$	6.4	3 and 52	0.0009
TAS (days 48–51)	0.57 ± 0.141$^{A, B}$	0.69 ± 0.229^{B}	0.50 ± 0.144^{A}	0.52 ± 0.117^{A}	3.7	3 and 53	0.02
Carotenoid concentration (day 58)	22.9 ± 3.93^{A}	25.0 ± 7.89^{A}	27.2 ± 4.76$^{A, B}$	32.2 ± 8.08^{B}	5.6	3 and 54	0.002
Log heterophil concentration (day 58)	1.23 ± 0.714	1.66 ± 1.072	1.33 ± 0.720	0.86 ± 0.547	2.4	3 and 51	0.08
Log lymphocyte concentration (day 58)	2.23 ± 0.455	2.62 ± 0.645	2.28 ± 0.633	2.01 ± 0.568	2.7	3 and 51	0.06
Log H/L (day 58)	0.30 ± 0.219	0.36 ± 0.346	0.30 ± 0.181	0.18 ± 0.092	1.4	3 and 51	0.24
Log WBC (day 58)	2.56 ± 0.478$^{A, B}$	2.99 ± 0.719^{A}	2.57 ± 0.662$^{A, B}$	2.21 ± 0.615^{B}	3.6	3 and 51	0.02
Humoral immune response to SRBC (day 68)	4.53 ± 2.200	4.33 ± 1.988	4.36 ± 1.646	3.93 ± 1.639	0.3	3 and 54	0.85
PHA swelling (day 69)	0.72 ± 0.221	0.62 ± 0.236	0.72 ± 0.266	0.72 ± 0.180	0.7	3 and 54	0.56

effect of coccidians on body condition, molt speed, or molt duration of House Sparrows. By contrast, we found that coccidians significantly and negatively affected feather quality during post-nuptial molt because infected birds had shorter wings and feathers that weighed less. These results contradict our previous report (Pap et al. 2009) that parasite manipulation had no effect on wing length of House Sparrows ($F = 0.7$, df = 1 and 45, $P = 0.40$). The negative effect of chronic coccidian infection is perhaps best explained by parasite-induced changes in protein uptake and absorption. Upon infection, sporozoites from the coccidian eggs burrow into the bowel wall, where they undergo several life cycle stages before rupturing through the fine lining of the bowel as unsporulated oocysts. At that time, the oocysts physically damage the bowel wall, resulting in a loss of blood and tissue fluid containing protein (Greiner 2008). As feather synthesis depends on protein availability (Murphy and King 1982, Pap et al. 2008), coccidian infestation has the potential to significantly affect feather growth. Coccidian infestation may also increase physiological stress and, ultimately, energy use by individuals. For instance, H/L ratio (an indicator of stress; Davis et al. 2008) was positively associated with infestation.

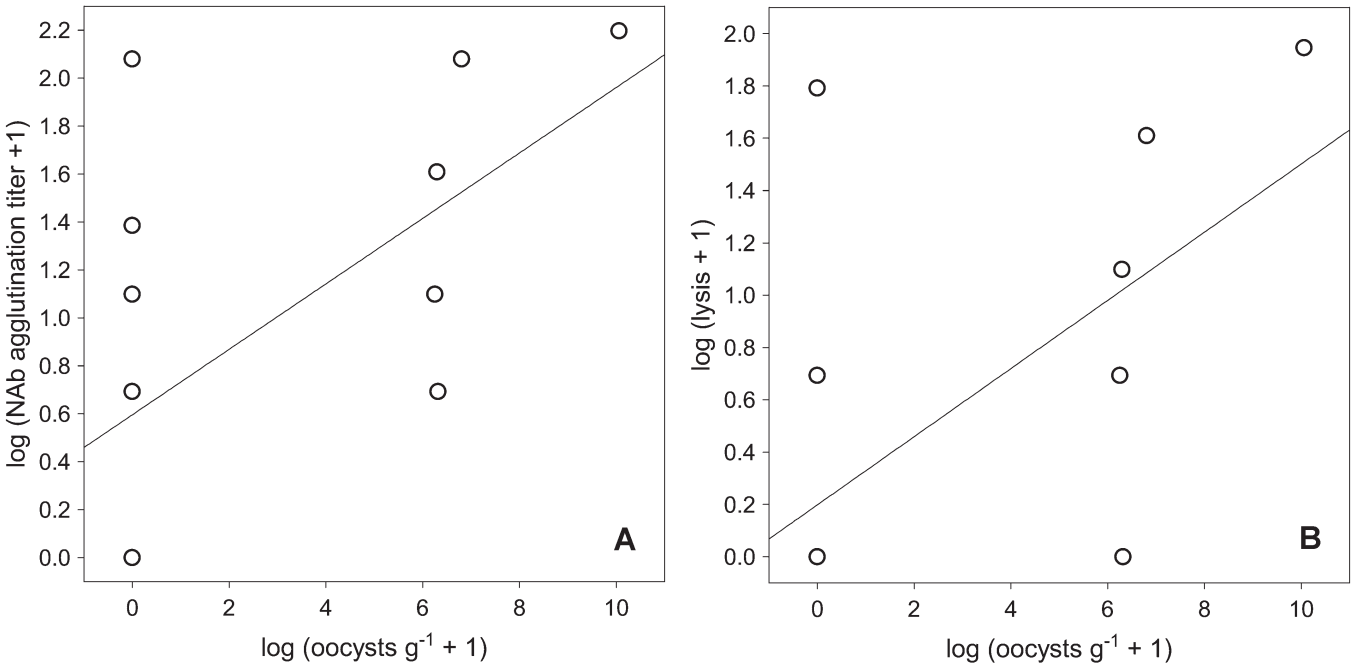

Fig. 5. The relationship between (A) NAb agglutination titer, (B) lysis and coccidian infestation of House Sparrows from Cocc groups measured at day 28 (data for Cocc2000 and Cocc5000 groups were pooled). The line is the least squares linear regression with the equation $y = 0.60 + 0.14\ x$, $r^2 = 0.35$, $P < 0.01$ (y = agglutination titer) and $y = 0.20 + 0.13\ x$, $r^2 = 0.38$, $P < 0.0001$ (y = lysis).

Regardless of the mechanisms involved, coccidians have the potential to significantly impair the flight capacity of the birds by altering wing loading (Norberg 2002). Consequently, infection can increase the energetic cost of flight and has the potential to reduce survival of infected individuals, and hence, fitness.

Our results also show that chronic coccidian infection stimulated several components of the constitutive immune system. Specifically, we observed a significant increase in the bactericidal activity of the whole blood against *E. coli* and *S. aureus* in infected individuals, and a significantly higher concentration of WBC in Cocc5000 in comparison to the Cont2 group. Furthermore, we found a positive association between infection intensity and both H/L ratio at capture, and agglutination and lysis measured at day 28. These immunological measures characterize the constitutive innate immunity (Matson et al. 2005, Tieleman et al. 2005, Buehler et al. 2008a), which is composed of a mixture of humoral and cellular components and provides the first line of immune defense against invading microorganisms. Thus, the increased bactericidal activity of the blood in Cocc groups and a significantly higher concentration of WBC in Cocc5000 in comparison to Cont2 group suggest that infection stimulated some components of the innate immune system (e.g., natural antibodies, complement, acute phase proteins, macrophages, heterophils and/or other immune cells). However, the effect on WBC should be treated with caution given the high number of multiple comparisons in this study and a significance level set at 0.05, which increases the probability of committing a type I error. We cannot rule out an alternative explanation that the anticoccidial drug suppressed the immune system of Cont birds, resulting in a lower bactericidal activity of the blood and suppression of the production of white blood cells of Cont birds relative to Cocc birds. However, we suggest that our results were only slightly affected, if at all, by the toltrazuril for the following reasons. First, toltrazuril has been shown to have negligible adverse effects on the immune system of poultry (Krautwald-Junghanns et al. 2009). Second, Cocc groups were treated with toltrazuril only once (at the beginning of the experiment), and the time elapsed between treatment and blood sampling for bacteria killing (i.e., 40 days) is likely to be a sufficient period for immune system recovery. Finally, under the assumption that toltrazuril suppresses the immune system, we would expect to see a difference in immune function between the two Cont groups because of the different dosages. This was not the case, because none of the physiological measures differed significantly between Cont1 and Cont2 groups.

Because the primary function of the immune system is defense, the first step in understanding the role of immunocompetence in host–parasite evolution is to determine the function of different components of immunity in providing protection against specific parasite challenges (Adamo 2004). Current knowledge concerning physiological defense against coccidians is primarily derived from the poultry literature, which is confounded by the effects of inbreeding and artificial selection and a focus on different parasite species (i.e., *Eimeria* coccidians; Allen and Fetterer 2002). Consequently, extrapolation of these results to free-living passerine birds, which are more commonly infested by *Isospora* coccidians, is limited. Our results support the findings of Whiteman et al. (2006) and Parejo and Silva (2009), who showed a significant association between parasites and both agglutination and lysis of avian blood. Future studies that relate these immunological measures to specific parasitic infections using common and reliable techniques to characterize the immune system (e.g., Tieleman et al. 2005, Matson et al. 2006, Buehler et al. 2008a, Forsman et al. 2008) will help broaden our understanding of host–parasite evolution. For instance, once the specific immune branches responsible for defense against coccidian infestations have been identified, the relative contributions of specific immune cell types (e.g., CD4, CD5 and CD8 lymphocyte lineages) could be determined by selectively depleting cells with monoclonal antibodies (Trout and Lillehoj 1996).

We also found that the PHA swelling response and lymphocyte concentration were not affected by chronic coccidian infestation, which is consistent with our previous study (Pap et al. 2009), but in contrast to other findings (Allen and Fetterer 2002, Hõrak et al. 2004, Saks et al. 2006, Mougeot et al. 2009). Recently, however, Lemus et al. (2010) questioned the use of PHA swelling as a measure of T-cell immune response against coccidian infection, which may explain the variability observed among these studies. Additionally, and in contrast to our previous study (Pap et al. 2009), SRBC-induced humoral immune response was not affected by coccidians. The inconsistency in the relationship between immune measures and chronic coccidian infection between studies, even within the same host–parasite system, same host population, and under similar environmental conditions, suggests that there may be a degree of plasticity in avian defense against coccidians or that potential difference between years in the physiological condition of the birds can alter immune defenses. Alternatively, the absence of significant difference between Cocc and Cont groups in PHA swelling, SRBC immune response, agglutination, lysis and differential leukocyte count may be explained by the down-regulation of the immune system during chronic infection, when most of our physiological measures were performed, relative to the acute infestation stage. During the acute infestation stage, immunological defense is upregulated, which would allow for easier detection of associations between specific immune cells and coccidians infestation.

In Red Grouse (*Lagopus lagopus scoticus*), nematode infection did not affect the TAS activity of the plasma (Mougeot et al. 2010). By contrast, our results in House Sparrows suggest that coccidian infestation increased TAS activity of plasma, which may indicate that immune system activation leads to increased free radical scavenging capacity of plasma to fight infection (Hőrak et al. 2007, Costantini and Møller 2009). The increase in TAS of infected birds might suggest that coccidians do not cause increased levels of oxidative stress. Alternatively, the increased TAS of infected birds may actually indicate the activation of the antioxidant system due to an increase in the levels of coccidian-induced oxidative stress. However, as mentioned above, this result and subsequent interpretation should be treated with caution given the high number of multiple comparisons and a significance level set at 0.05, which increases the probability of committing a type I error.

Interestingly, carotenoid concentration, which may influence immunostimulation and regulation of oxidative processes (Møller et al. 2000; but see Costantini and Møller 2008), was significantly reduced in the infected birds compared to the Cont2 group, indicating the possible depletion or the disruption of the absorption of these biomolecules following infection. However, the absence of a relationship between TAS and carotenoid concentration suggests that antioxidant capacity was not influenced by the levels of circulating carotenoids.

Although the negative effects of antihelminthic and anticoccidial drugs on the immune system are well documented (Sajid et al. 2006), we are not aware of any studies of wild birds that examined the effect of endoparasites with joint control of the confounding effects of these drugs. We found no significant difference between Cont groups in any of the morphological, physiological or immunological measures used in this study, which suggests that our results were not confounded by the effects (either positive or negative) of the anticoccidial drug (see also Krautwald-Junghanns et al. 2009 for the Domestic Pigeon [*Columba livia domestica*]). Furthermore, following infestation by coccidians, the intensity of parasitism between Cont groups was similar, indicating that a doubling of the anticoccidial drug did not provide additional protection against infestation. Therefore, in line with previous findings for this drug (Krautwald-Junghanns et al. 2009, Pap et al. 2009), we suggest that concentration of 25 mg toltrazuril (i.e., 1 mL of Baycox in 1 L drinking water) is sufficient for purging the parasites. However, regardless of the anticoccidial drug used, we were unable to fully eliminate the coccidian infestation as birds were continuously reinfested from the environment. Finally, we have shown that under aviary conditions, where the birds can be reinfected by ingesting oocysts found in their environment, the infection dose had no affect on the subsequent chronic infection intensity of the birds (see also Filipiak et al. 2009). This conclusion is also suggested by the absence of a significant difference in condition and physiological and immunological measures between Cocc2000 and Cocc5000 groups. Nonetheless, the lack of difference between Cocc groups does not exclude the possibility of an effect of infection dose on acute infection intensity of the birds.

In conclusion, we found that chronic infestation by *Isospora* coccidians had a negative effect on the condition of the House Sparrows because it reduced the length and quality of flight feathers grown during molt. This effect may be attributed to coccidian–host competition for protein sources or by an increase in energy use because of infection (indicated by the increased H/L ratio). Furthermore, both the increased bactericidal activity of the blood and the association between coccidian infestation and agglutination level and lysis against rat blood cells suggests that coccidians stimulate several aspects of the constitutive innate immune system in birds.

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