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Published in:
Physiological and Biochemical Zoology

DOI:
10.1086/676934

No Evidence for Parasitism-Linked Changes in Immune Function or Oxidative Physiology over the Annual Cycle of an Avian Species

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Accepted 3/13/2014; Electronically Published 8/15/2014

ABSTRACT

Temporally changing environmental conditions occur in most parts of the world and can exert strong pressure on the immune defense of organisms. Seasonality may result in changes in physiological traits over the year, and such changes may be essential for the optimization of defense against infections. Evidence from field and laboratory studies suggest the existence of links between environmental conditions, such as infection risk, and the ability of animals to mount an immune response or to overcome infections; however, the importance of parasites in mediating seasonal change in immune defense is still debated. In this study, we test the hypothesis that seasonal change in immune function and connected physiological traits is related to parasite infection. We sampled captive house sparrows (*Passer domesticus*) once every 2 mo over 14 mo and compared the annual variation in 12 measures of condition, immune function, antioxidant status, and oxidative damage among birds naturally infested with coccidians or medicated against these parasites. We found significant variation in 10 of 12 traits over the year. However, we found little support for parasite-mediated change in immune function and oxidative status in captive house sparrows. Of the 12 measures, only one was slightly affected by parasite treatment. In support of the absence of any effect of coccidians on the annual profile of the condition and physiological traits, we found no consistent relationships between the intensity of infestation and these response variables over the year. Our results show that chronic coccidian infections have limited effect on the seasonal changing of physiological traits and that the patterns of these measures are probably more affected by acute infection and/or virulent parasite strains.

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Physiological and Biochemical Zoology 87(5):729–739. 2014. 1522-2152/2014/8705-3155$15.00. DOI: 10.1086/676934

Introduction

Temporally changing environmental conditions occur in most parts of the world and can exert strong pressure on the immune defense of organisms. Seasonality may result in changes in physiological traits over the year, and such changes may be essential for the optimization of defense against infections (Nelson et al. 2002; Møller et al. 2003; Hasselquist 2007; Buehler et al. 2008*a*; Martin et al. 2008). Evidence from field and laboratory studies suggests the existence of links between environmental conditions, such as food availability, temperature, and infection risk, and the ability of animals to mount an immune response or overcome infections (Møller et al. 2003; Buehler et al. 2008*a*, 2009; Hawley and Altizer 2011; Martin et al. 2011). However, the importance of these factors in mediating seasonal change in immune defense is still debated (e.g., Buehler et al. 2009; Hawley and Altizer 2011; Horrocks et al. 2012). Thus, investigating the seasonal dynamics of an organism's immune response under various environmental conditions offers a better understanding of temporal variation in resistance to parasites and pathogens. Controlled studies that manipulate external settings are needed to tease apart the relative importance of different contributors to patterns of immune variation.

Studies of the impact of environmental conditions on physiological defense over a complete annual cycle are limited, and the results are ambiguous. Although studies of birds and mammals indicate that seasonal variation in immune function is the rule rather than the exception (Nelson et al. 2002; Møller et al. 2003; Buehler et al. 2008*a*; Martin et al. 2008), some recent studies suggest that some components of the immune system may remain relatively constant over the year (Hegemann et al. 2012*b*, 2013). Furthermore, manipulating different aspects of the environment results in different effects on immune func-

tion. Temperature manipulation resulted in no effect on indexes of immune function (Buehler et al. 2008*a*); however, food limitation proved to be of major importance for at least some components of the immune response even over a short period of limitation (Buehler et al. 2009). Finally, parasites and infectious diseases are considered one of the major selective agents acting on the immune defense of animals (Møller et al. 2003; Hawley and Altizer 2011), yet no study, to the best of our knowledge, has addressed changes in immune function triggered by parasite infections through the year. This omission may be explained by the difficulty of manipulating the parasite load of the host over a long period and of repeatedly measuring infection load and the physiological response to the treatment.

Studies of wild and captive animals are important when unraveling relationships between ecology and physiology. However, the results of field studies tend to be too complex to interpret mechanistically, particularly if confounding factors, such as temperature, access to food resources, and infection by parasites and microbes, are difficult to control. Such confounding factors are likely to be important for both the immune system and its associated antioxidant system (see below; Monaghan et al. 2009; Hasselquist and Nilsson 2012). There are different ways to investigate variation in the immune function over the annual cycle. For example, one can sample different individuals from the same (local) free-living population on several occasions. Alternatively, one can measure the same random sample of the population using repeated measurements of individuals. The first approach allows studying the physiological response of individuals to environmental factors under natural conditions, but it lacks detailed longitudinal knowledge about the sampled individuals (e.g., Pap et al. 2010; Horrocks et al. 2012). The second approach allows studying the immune function in response to experimental manipulation of environmental factors through the annual cycle while other factors are standardized (e.g., Buehler et al. 2008*a*, 2012; Versteegh et al. 2012*a*). However, due to the logistical constraints of capturing the same individuals for repeated measurements and manipulation of parasite load, this approach is workable almost exclusively under captive conditions.

We used the second approach and sampled our study organism, the house sparrow (*Passer domesticus*), once every 2 mo. In addition, infection of birds with a common unicellular endoparasite, the *Isospora* coccidians, could be followed through the annual cycle, and the parasite load could be manipulated in relation to the control group. This allowed us to study the effect of parasite infection on physiology over the whole year. We measured six immunological variables to characterize the immune system and to study its response to infection over the annual cycle (leukocyte counts: heterophils, lymphocytes, and total leukocytes; activity of natural antibodies and the complement system: hemolysis and hemagglutination; lysozyme level). We also measured two components of the antioxidant system (total antioxidant status [TAS] and the concentration of uric acid [UA]) and evaluated oxidative damage on the membrane lipids (concentration of malondialdehyde [MDA]) because immune system activation can affect the antioxidant system (Costantini and Møller 2009; Monaghan et al. 2009; Sorci and Faivre 2009). All these variables of immune function and oxidative status (except lysozyme) have been shown to be affected by coccidian infection in wild birds (Hõrak et al. 2004; Pap et al. 2009, 2011; Sepp et al. 2012*a*; Bókony et al. 2014). This approach allowed us to test the hypothesis that seasonal change in immune function and connected physiological traits are related to level of parasite infection. We first examine how different indexes change over the annual cycle, and we present data on the putative effect of parasite manipulation on these changes. We also present the repeatability of physiological indexes measured over the year. We then analyze covariation between physiological traits and parasite load during each sampling period.

Material and Methods

Data Collection and Experimental Protocol

Thirty adult male and 30 adult female house sparrows were caught with mist nets (Ecotone, Gdynia, Poland) at a farm near Bălcaciu (46°10′N, 24°03′E), central Transylvania, Romania, just before the annual postnuptial molt on July 30, 2010. Birds were transported to the campus of Babeş-Bolyai University in Cluj Napoca and housed in four groups ($N = 15$ birds in each; seven males and eight females or, inversely, alternating among groups) in outdoor aviaries (5 m × 2 m × 2.5 m [length × width × height]) with the experimental treatments arranged alternately (two replicates for each treatment group; see below). Birds were fed ad lib with a mixture of seeds containing ground corn, sunflower, wheat, and oat throughout the experiment. In addition, every second day birds were given a protein supplement of two grated boiled eggs. Fresh tap water was provided daily. We sampled the birds over two consecutive days by the middle of every second month, starting in August 2010, between 8 a.m. and 2 p.m. The sampling order was randomized by aviary and individual between the eight measurement sessions. We collected about 250 μL of blood into heparinized capillary tubes within 20 min of entering the aviary at each capture. Immediately after sampling, we made blood smears and transported the remaining blood in cooling bags to the laboratory for further processing within 3 h of sampling.

Birds were allowed to acclimatize in aviaries for 2 wk. Afterward, on August 15, we placed the birds in individual cages to quantify the preexperimental level of coccidian infestation by measuring the rate of oocyst shedding over 2 d (for details, see Pap et al. 2009, 2011, 2013). In a previous study of the house sparrow, we demonstrated that *Isospora lacazei* was the most common coccidian in the fecal samples (Pap et al. 2011). Because 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 chambers, as described previously (Pap et al. 2011, 2013), and the concentration was expressed as the number of oocysts per gram of feces. The mean values of the oocyst numbers collected during the 2 d were used in the analyses to test variation in pathogen load, condition, and phys-

iological traits over the annual cycle and the effect of coccidian treatment on measures of the 12 traits (see "Statistical Analyses"). The next day (August 17), we randomly assigned birds to one of the four aviaries and each aviary to either (1) medication with an anticoccidial drug (the medication group; $N = 30$) or (2) natural infestation with coccidia (the infestation group; $N = 30$; i.e., we had two replicates for each treatment group). We treated the medicated birds against coccidians 2 d per week through the experiment (Baycox, Bayer Healthcare, Germany; 2.5 g toltrazuril in 100 mL^{-1} water) because, under aviary conditions, the reinfestation of birds from the environment is highly probable (P.L.P., C.I.V., personal observation), making it necessary to continuously suppress the reproduction of these parasites. Our previous study shows that the drug used in the present experiment has no negative side effects on the condition and physiology of house sparrows (Pap et al. 2011). Birds in the infestation group received tap water, which allowed the coccidians to persist in the alimentary tract (fig. 1*A*).

Some house sparrows built nests, laid eggs, and even raised chicks during the breeding season, although the identity of birds with nests was not determined. The timing and duration of postnuptial molt, presented in a previous study (Pap et al. 2013), shows that house sparrows in captivity retain molt schedules and the annual routine. Of the 60 captured house sparrows, four individuals died of unknown causes (three from the medication group and one from the infestation group). This low mortality rate over such a long period of experimentation proves that birds acclimatized well. All other individuals were released in good health at the end of the experiment on November 3, 2011.

Measuring Immune Function and Oxidative Stress

Hematocrit. Bleeding was performed using 75-μL heparinized capillary tubes by venipuncture of the brachial vein directly after capture. Blood was centrifuged for 5 min at 6,200 *g*, and hematocrit was measured as the fraction of red blood cells in the whole blood sample. The plasma and packed cell fractions were then separated and stored at −20°C until further analyses (samples were stored 6–12 mo depending on measure, and samples obtained at different time periods [months] were randomized before analyses).

Leukocyte Count. A drop of blood was smeared on a microscope slide, air-dried, fixed in Dia-Fix, and stained with Dia-Red and Dia-Blue Panoptic (Diagon, Budapest, 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 (fewer than 3 cells per 10,000 erythrocytes). Leukocytes were counted by the same person (A.S.) and were moderately to highly repeatable (heterophils: intraclass correlation coefficient [ICC] = 0.46, 95% confidence interval [CI] = 0.05–0.87, $F_{14, 15} = 2.70$, $P = 0.03$; lymphocytes: ICC = 0.69, 95% CI = 0.42–0.96, $F_{14, 15} = 5.50$, $P = 0.001$; total leukocytes: ICC = 0.55, 95% CI = 0.20–0.91, $F_{14, 15} = 3.47$, $P = 0.01$; heterophil-to-lymphocyte (H : L) ratio: ICC = 0.90, 95% CI = 0.79–1.00, $F_{14, 15} = 18.00$, $P < 0.0001$).

Hemolysis-Hemagglutination Assay. The constitutive humoral immunity (i.e., the levels of the natural antibodies and complement) was assessed using a modified hemolysis-hemagglutination assay (Matson et al. 2005; Pap et al. 2010), described in detail elsewhere (Pap et al. 2010). The only modification related to our previous work was that here we used a commercial 2% rabbit red blood cell suspension (Biotrend Chemikalien, Cologne, Germany). In this assay, agglutination reflects the activity of the natural antibodies, while lysis represents the interaction between the natural antibodies and the complement (Matson et al. 2005; Buehler et al. 2008*a*).

Lysozyme. Lysozyme is a broadly effective antimicrobial enzyme expressed by macrophages and has therefore been used as a stage-specific marker (Davison et al. 2008). Lysozyme concentration in plasma was measured using the lysoplate assay method (Osserman and Lawlor 1966) with slight modification. In brief, 10 μL of plasma was placed in the test wells of a 1% agar gel (Sigma-Aldrich, St. Louis, MO), containing 50 mg/100 mL lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich). Plates were incubated at 37°C for 22 h. In this assay, bacterial lysis occurs as a clear zone developed in the area of the agar surrounding the wells. The diameters of the cleared zones are proportional to the $\log_{10}$ of lysozyme concentration. This area was measured using digital calipers, and lysozyme concentration (μg/mL) was determined on the basis of a standard curve, performed using lysozyme from chicken egg white (Sigma-Aldrich). The repeatability of a subsample measured twice was moderate but significant (ICC = 0.66, 95% CI = 0.31–1.00, $F_{9, 10} = 4.95$, $P = 0.01$).

TAS. TAS is a composite measure of antioxidant capacity, expressing the cumulative ability of all nonenzymatic antioxidants found in plasma, such as vitamins, sulfhydryl groups of proteins, and UA, to combat a simulated free radical insult. TAS was measured colorimetrically from 5 μL of plasma, using the TAS kit (Cayman Chemical, Ann Arbor, MI) as described previously (Bókony et al. 2014). This assay relies on the ability of antioxidants in the plasma to inhibit the formation of $ABTS^+$ from oxidation of ABTS (2.2′-azino-di-(3-ethylbenz-thiazoline sulfonate)) by metmyoglobin. An antioxidant of known concentration (Trolox) was used as a standard for the calculation of antioxidant levels in the samples. Values of TAS are expressed as mM/L Trolox equivalents. The repeatability of a subsample measured twice was moderate but significant (ICC = 0.54, 95% CI = 0.034–0.832, $F_{12, 13} = 3.37$, $P = 0.019$). Since UA is a component of TAS as well as a product of amino acid catabolism, we controlled TAS for UA levels by ordinary least squares regression and calculated residual TAS as suggested by

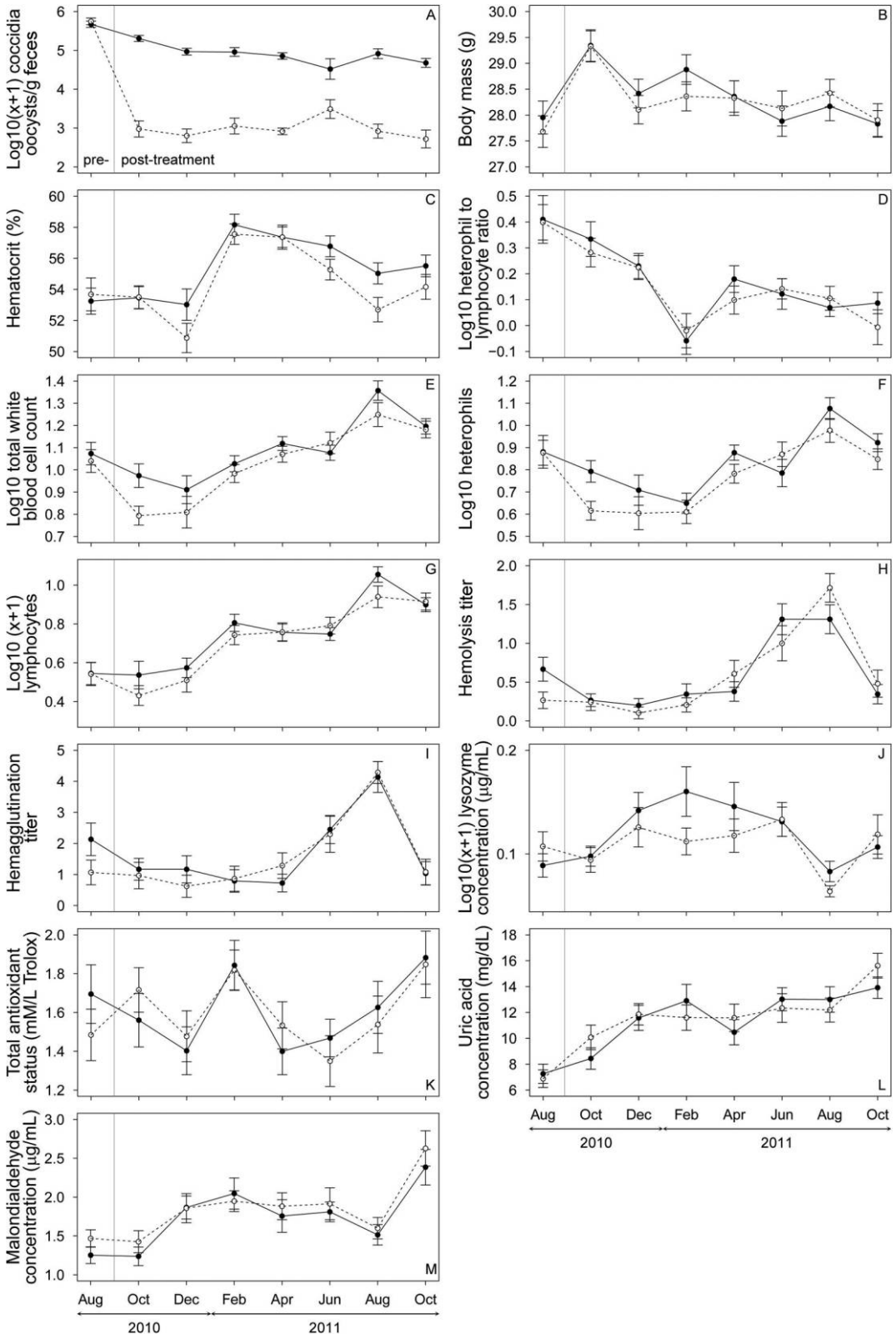

Figure 1. Trends in coccidian infestation (*A*), body mass (*B*), hematocrit (*C*), constitutive immunity (*D–J*), and antioxidant capacity and oxidative stress (*K–M*) throughout the annual cycle. Open and filled circles indicate medication and infestation treatments, respectively. Symbols represent means, and error bars show 1 SE. Because of repeated measures, error bars can be used only to infer statistical differences between treatments, not month-to-month differences.

Cohen et al. (2007). Results for residual TAS were highly similar to those for raw TAS and are omitted throughout.

UA. Plasma UA concentration was determined from 5 μL of plasma using a spectrophotometrically uricase/peroxidase method (Uric Acid Liquicolor Kit; Human, Wiesbaden, Germany). Results are given in milligrams per deciliter of plasma (see Bókony et al. 2014). Repeatability of duplicate measures was very high (ICC = 0.99, 95% CI = 0.965–0.995, $F_{16, 17}$ = 15.3, $P < 0.001$).

MDA. MDA is a carbonyl compound that results from the peroxidative degeneration of membrane polyunsaturated fatty acids by reactive oxygen species; thus, it is a widely used marker of oxidative stress (Del Rio et al. 2005). MDA concentration was determined from 10 μL of plasma by high-performance liquid chromatography (HPLC) on a HPLC SUPELCOSIL LC-18 column (5-μm particle size; Sigma-Aldrich) with UV detection at 254 nm (Jasco UV-2075 Plus; Tokyo, Japan; Bókony et al. 2014). The mobile phase was 30 mM monopotassium phosphate (KH_2PO_4)–methanol (65 : 35, v/v %), and the flow rate was 0.5 mL/min. The retention time of MDA recorded was around 6 min. MDA concentration in the sample was determined using a calibration curve (R^2 = 0.99) of a series of standards generated by acidic hydrolysis of 1,1,3,3-tetraethoxypropane (Sigma-Aldrich). Results are given in micrograms per milliliter of plasma and are not corrected for the dilution factor (see Bókony et al. 2014). The repeatability of the subsample measured twice was very high (ICC = 0.97, 95% CI = 0.911–0.989, $F_{14, 15}$ = 62.2, $P < 0.001$).

Statistical Analyses

We chose to employ the frequentist (null hypothesis significance testing) paradigm because such statistics provide well-established, efficient statistical tests for data collected under experimentally controlled or manipulated conditions (Pinheiro and Bates 2000) where the goal is to understand the effect of specific variables and manipulations (Richards et al. 2011).

Repeatabilities at the individual level based on repeated measures were calculated using the following equation: R = variance$_{among}$/(variance$_{within}$ + variance$_{among}$) (Lessels and Boag 1987). Among- and within-individual variances were acquired using the VarCorr command from the linear mixed-effects model (lme) function in the package nlme (Pinheiro and Bates 2000). For repeatability calculations, we used null models that included only individual as a random factor and did not include fixed effects. For the repeatability analysis, we included only samplings that took place after the experimental treatment had started (N = 7 samplings) and individuals for which we had data from all of those samplings (birds = 56).

We used linear mixed-effects models to test for fixed (experimental group, month, sex) and random (aviary, individual) effects on measures of condition, pathogen load, immune function, and oxidative physiology (the lme function in the package nlme; Pinheiro and Bates 2000). For these analyses, we used all birds and all samplings. The nesting structure was such that individual birds were nested within aviaries, and birds were sampled repeatedly over several months. We began with full models that included all fixed and random effects as well as the second-order interactions between group, month, and sex and then removed the interactions and the variable sex from the models if these terms were not significant at the P = 0.05 level.

To calculate effect sizes, we used equations (10) and (11) presented in Nakagawa and Cuthill (2007) to calculate Cohen's d for the categorical fixed variables and r for the continuous variable month and interactions containing month, respectively. We retrieved t values and degrees of freedom from the summary output of models. If an interaction or variable was removed from the final model because it did not reach statistical significance at the P = 0.05 level, then effect size for that term was calculated using summary data from the last model that included the variable in question.

The residuals of all models were inspected visually to verify the normality of the distributions using histograms, Q-Q plots, and residual versus fitted plots. We used $\log_{10}(x + 1)$ transformation on the number of oocytes per gram of feces, heterophils, lymphocytes, H : L ratio, total white blood cell count, and lysozyme. These transformations resulted in normally distributed residuals. No transformation was helpful for normalizing residuals for models of lysis; those residuals remained slightly skewed. All other variables conformed to normality without transformation. All statistical analyses and graphing were performed in the R programming environment (R Development Core Team 2012).

Results

All condition and immune variables and measures of antioxidant system and oxidative damage except lysozyme concentration and TAS varied significantly over the year (fig. 1*B*–1*M*, table 1); however, the pattern depended on measures. All but two measures were similar between the sexes, as revealed by the nonsignificant effect of sex and the sex × treatment interaction ($F < 3.55$, $P > 0.065$; table 1). Males were heavier and had higher hemolysis scores than females.

The anticoccidial drug significantly reduced the number of oocysts shed in feces (fig. 1*A*, table 1), as revealed by the significant effect of treatment and the significant month × treatment interaction when the data on the pretreatment sample were included in the analysis ($r = -0.14$, $F_{1, 385} = 7.82$, P = 0.005). Coccidian infestation significantly changed over the year (table 1), and the number of oocysts steadily decreased in the infestation group during the course of the experiment (fig. 1*A*). The infestation rate and the success of medication cure were similar between sexes, as revealed by the nonsignificant effect of sex and the sex × treatment interaction ($F < 2.27$, $P > 0.137$). Medication had no significant effect on any of the condition indexes, immune variables, or measures of antioxidant system and oxidative damage, as revealed by the nonsignificant effect of treatment ($F < 4.21$, $P > 0.176$; table 1) and, except

Table 1: Fixed and random effects on variation in condition, pathogen load, immune function, and oxidative stress in house sparrows over the annual cycle

	Among individuals																
	Treatment, fixed				Sex, fixed				Aviary, random			Bird within aviary, random			Within individuals, month, fixed			
	df	*F*	*P*	*d*	df	*F*	*P*	*d*	df	*L* ratio	*P*	df	*L* ratio	*P*	df	*F*	*P*	*r*
Condition and stress:																		
Body mass	1, 2	.2	.70	−.6	1, 55	7.8	**.01**	**−.8**	1, 55	<.001	1.00	1, 55	426.3	**<.001**	1, 402	21.3	**<.001**	**−.21**
Hematocrit	1, 2	.7	.49	−1.2	1, 55	3.6	.07	−.5	1, 55	2.1	.14	1, 55	73.8	**<.001**	1, 402	13.5	**<.001**	**.18**
Heterophil-to-lymphocyte ratio	1, 2	.2	.68	−.7	1, 54	.1	.74	−.1	1, 54	1.8	1.00	1, 54	17.4	**<.001**	1, 394	54.1	**<.001**	**−.35**
Pathogen load:																		
Coccidian infestation	1, 2	71.9	**.01**	**–12.0**	1, 54	2.3	.14	.41	1, 55	<.001	1.00	1, 55	.2	.70	1, 336	7.6	**.006**	**−.15**
Immune function:																		
Heterophils	1, 2	2.4	.26	−2.2	1, 54	.1	.72	−.1	1, 54	.6	.46	1, 54	.4	.56	1, 394	20.8	**<.001**	**.22**
Lymphocytes	1, 2	1.7	.33	−1.8	1, 54	.0	1.00	−.0	1, 55	<.001	1.00	1, 54	1.1	.28	1, 394	154.6	**<.001**	**.53**
Total white blood cells	1, 2	4.2	.18	−2.9	1, 54	.5	.50	−.2	1, 54	.2	.69	1, 54	2.1	1.00	1, 394	70.8	**<.001**	**.39**
Hemolysis	1, 2	3.5	.20	−2.7	1, 55	5.3	**.03**	**−.6**	1, 55	.4	.56	1, 55	8.3	**<.01**	1, 398	4.7	**.03**	**.16**
Hemagglutination	1, 2	.1	.79	−.4	1, 55	2.2	.14	−.4	1, 55	.1	.71	1, 55	49.7	**<.001**	1, 399	21.7	**<.001**	**.23**
Lysozyme	1, 2	.6	.54	−1.1	1, 55	.0	1.00	−.0	1, 55	.8	.38	1, 55	7.7	**<.01**	1, 401	.0	.96	−.00
Oxidative stress:																		
Total antioxidant status	1, 2	.0	.89	−.2	1, 55	.1	.80	−.1	1, 55	<.001	1.00	1, 55	5.3	**.02**	1, 386	1.1	.29	.05
Uric acid	1, 2	.1	.80	.4	1, 54	.5	.50	.19	1, 54	<.001	1.00	1, 54	22.0	**<.001**	1, 398	77.9	**<.001**	**.41**
Malondialdehyde	1, 2	.7	.50	1.2	1, 54	1.5	.23	.33	1, 54	<.001	1.00	1, 55	11.1	**<.001**	1, 393	35.4	**<.001**	**.29**

Note. Note that in the case of coccidian infestation the data from pretreatment (August 2010) are excluded (for details, see the text). Test statistics for linear mixed-effect models and associated effect sizes (Cohen's *d* and *r*) are shown. Significant interactions were observed in the case of hemolysis (treatment × month: $F = 4.68$, df = 1, 398, $r = 0.11$, $P = 0.03$). Boldface type indicates statistical significance.

for hemolysis, the nonsignificant month × treatment effect ($F < 4.21$, $P > 0.176$). This shows that only one measure changed over the year as a function of coccidian infestation.

All condition indexes, immune variables, and measures of antioxidant system and oxidative damage except leukocyte counts and TAS were low to highly repeatable within individuals over the annual cycle. Repeatabilities varied between 0.07 and 0.78 (table 2).

We used bivariate correlations to examine the consistency of interseasonal correlations at the month level. The ellipse plots clearly show generally low and inconsistent correlations between coccidian infestation and condition indexes, immune variables, and measures of antioxidant system and oxidative damage over the annual cycle in the medication and infestation groups and in males and females (fig. 2).

Table 2: Individual repeatability of immune function in house sparrows over the annual cycle

	R	df	*L* ratio	*P*
Condition and stress:				
Body mass	.78	1	404.8	**<.001**
Hematocrit	.31	1	63.4	**<.001**
Heterophil-to-lymphocyte ratio	.11	1	10.5	**.001**
Pathogen load:				
Coccidian infestation	.53	1	216.9	**<.001**
Immune function:				
Heterophils	.01	1	.2	.690
Lymphocytes	<.001	1	<.001	1.000
Total leukocytes	<.001	1	<.001	1.000
Hemolysis	.07	1	4.6	**.032**
Hemagglutination	.21	1	33.8	**<.001**
Lysozyme	.11	1	10.1	**.002**
Oxidative stress:				
Total antioxidant status	.06	1	2.9	.090
Uric acid	.15	1	18.2	**<.001**
Malondialdehyde	.10	1	8.9	**.003**

Note. The significance of repeatability (*R*) was determined by contrasting models with and without individual as a random factor, using likelihood ratio tests. Note that the measures before the coccidian treatment are excluded from these analyses. Boldface type indicates statistical significance.

Discussion

Effect of Coccidians on Condition and Physiological Measures over the Year

We found little support for a parasite-mediated change in immune functions and oxidative status in captive house sparrows. The parasite exposure hypothesis has been suggested as one possible explanation for the seasonal changes in physiological traits (Nelson et al. 2002; Møller et al. 2003; Hasselquist 2007; Martin et al. 2008), but it was rarely tested on wild birds. Of the nine condition and immune variables and three markers of antioxidant capacity and oxidative damage studied by us, only one was affected by parasite treatment (significant treatment × month interaction in the case of hemolysis). In support of the absence of a treatment effect, we found no consistent relationships between the intensity of infestation and these response variables over the year (fig. 2). In general, the correlations were nonsignificant, and in the case of significant relationships within an experimental group or sex category, these changed to nonsignificance during the next sampling periods or in the case of a different group (experimental or sex category). The absence of any consistent effect of coccidians on condition and physiological traits is somewhat surprising since our medication was effective, producing a large and significant difference in the infestation rate between experimental groups through the year. It has been shown that coccidians have a mild but significant effect on the nutritional uptake, general physiological condition, and fitness of wild birds (Hõrak et al. 2004; Baeta et al. 2008; Pap et al. 2011, 2013). Coccidians stimulate the immune system of birds and its associated antioxidant machinery (Allen and Fetterer 2002; Greiner 2008; Pap et al. 2011; Sepp et al. 2012*a*), and all condition and physiological measures except lysozyme have been shown to be sensitive measures of coccidian infestation in wild birds, particularly in house sparrows (Hõrak et al. 2004; Pap et al. 2009, 2011; Sepp et al. 2012*a*; Bókony et al. 2014). It is worth mentioning that the infestation rate among birds in the infestation group and the effect of medication were similar to those in our previous experiments (Pap et al. 2009, 2011; note that in these studies the number of coccidians was $\log_e$-transformed). In both of these studies we found a significant effect of coccidians on the condition, immune response, and antioxidant status of house sparrows. Therefore, the intensity of infestation probably does not explain the lack of effect of parasite manipulation on the host's condition and physiological measures. We formerly demonstrated on the same set of house sparrows that the medication treatment clearly affected the condition of birds, at least during molting, which was supported by the significant negative effect of coccidians on the size of the uropygial gland and on the quality of flight feathers (Pap et al. 2013). These findings suggest that the lack of effect of coccidians in modulating the variation of the immune profile and oxidative status of house sparrows in this experiment is probably not confounded by low sample size, inefficient manipulation of the infestation rate, or a minimal effect on the host's condition.

Our results support the benign effect of chronic coccidian infestation on wild birds (see Greiner 2008), with a limited effect of these parasites on seasonal changes in physiological traits. This finding is also strongly supported by our recent work in free-living house sparrows, where under natural conditions chronic coccidian infestation was weakly associated with all of the physiological traits measured (heterophils, lymphocytes, total leukocyte counts, total antioxidant capacity, UA, and MDA; P. L. Pap, L. Pătraş, G. Osváth, D. M. Buehler, M. A. Versteegh, A. Sesarman, M. Banciu, and C. I. Vágási, unpublished data). However, the impact of coccidian infestation may be particularly severe during the acute phase of infection and during stressful periods in an animal's life (Allen and Fetterer 2002; Greiner 2008). In most cases where we have found immune traits and oxidative status to respond to coccidian

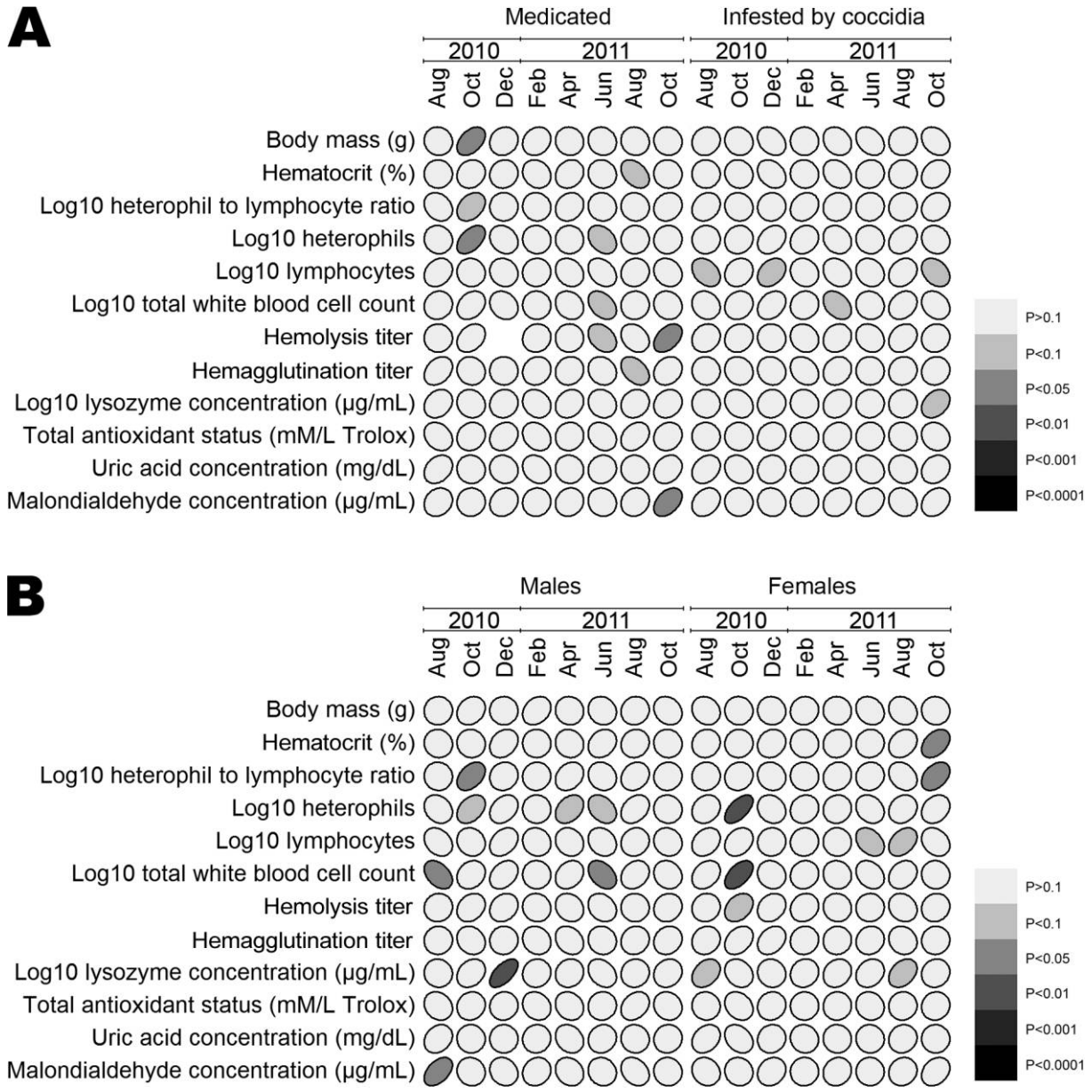

Figure 2. Pearson correlations between coccidian infestation (columns) and condition indexes, immune variables, and measures of antioxidant system and oxidative damage (rows) over an annual cycle for birds in the infestation and medication groups (*A*) and in males and females (*B*). Each ellipse represents a correlation from 27 and 29 birds in the infestation and medication groups, respectively (*A*), or 28 male and 28 female birds within a given month (*B*). In *A*, an ellipse is missing for infested birds in December 2010 because of highly zero-inflated hemolysis. Right-tilted ellipses indicate positive correlations, and left-tilted ellipses indicate negative correlations. The narrowness of ellipses is proportional to correlation *r* values, and shading is proportional to *P* values (see the key).

infestation, measurements were taken immediately following experimental infection; hence, these results refer to acute infection (e.g., Hõrak et al. 2004; Pap et al. 2009, 2011; Sepp et al. 2012*a*). Thus, the difference between acute and chronic infection may at least partially explain the reduced response of birds to coccidian infestation. However, we cannot exclude the possibility that the benign conditions experienced by the birds in captivity, including ad lib access to high-quality food and the lack of other constraints (such as predation), made it possible for the birds to operate immune system and oxidative physiology at the same level as medicated individuals because they were not resource and/or time limited.

Seasonal Change and Repeatability of Condition and Physiological Traits

We found significant variation in 10 of 12 condition and immune traits and measures of oxidative physiology over the year

(table 1), which supports previous results for seasonal changes in physiological traits in wild and captive birds (Romero 2002; Buehler et al. 2008*a*, 2012; Pap et al. 2010; Hegemann et al. 2012*a*, 2013; Horrocks et al. 2012; Versteegh et al. 2012*a*; but see Hegemann et al. 2012*b*). However, the pattern of change was inconsistent between traits, and, contrary to what has been found in house sparrows (Pap et al. 2010), males and females were similar in all but two measures (significant effect of sex on body mass and hemolysis). In a previous study of the same species sampled under natural conditions (Pap et al. 2010), we found that hemagglutination and hemolysis have at least partly different patterns of change over the year in comparison to what we found in the present study and that males and females differ at least during mating. In a more recent study of wild-living house sparrows, we found that, among immune variables, the number of different leukocyte types (e.g., lymphocytes and heterophils), total leukocyte counts, and H : L ratio and, among measures of oxidative status, total antioxidant capacity, UA, and MDA vary at least partly differently compared with what we have found in captive birds, and again the concentration of leukocytes in females is higher than that in males (P. L. Pap, L. Pătraş, G. Osváth, D. M. Buehler, M. A. Versteegh, A. Sesarman, M. Banciu, and C. I. Vágási, unpublished data). These results indicate that circumstances related to captivity may affect the change in physiological traits over the year (Buehler et al. 2008*b*; Sepp et al. 2010). In addition, the difference in annual patterns between sexes can be masked in captive compared with wild-living birds.

Physiological indexes are assumed to reflect fundamental attributes of individuals; however, the extent of variation over the year has been rarely tested (but see Buehler et al. 2008*a*; Norte et al. 2008; Matson et al. 2012). We found that the repeatability of physiological measures was generally low but statistically significant or low and nonsignificant, indicating low consistency within individuals over the year. The repeatability of hemagglutination and hemolysis scores is similar to what has been found in red knots (*Calidris canutus*) under similar experimental conditions (Buehler et al. 2008*a*), but the values for differential and total leukocyte concentrations measured by us were very low and nonsignificant compared with those in the formerly mentioned study and those that have been found in great tits (*Parus major*; Norte et al. 2008). The low consistency of the physiological traits within individuals—in particular, of the leukocyte concentrations, hemolysis, and TAS—might be explained by measurement error; however, the high repeatability values between repeated measures of the same samples (see "Material and Methods") exclude this possibility. Our results show that physiological traits can change flexibly within individuals over the year, which can be explained by the redistribution of immune cells and measures of oxidative status between body fluids and organs (see Buehler et al. 2008*a*; Kuhlman and Martin 2010; Sepp et al. 2012*b*). In addition, consistency within individuals may change through the year as well, and as time series increase in length and include more consecutive samples, the repeatability of physiological traits may decline (Norte et al. 2008; Matson et al. 2012). These effects may further explain the low repeatability values we found. The loose links among physiological traits at the species, population, and individual level (Forsman et al. 2008; Cohen and McGraw 2009; Buehler et al. 2011, 2012; Sepp et al. 2012*b*; Versteegh et al. 2012*b*) suggest that these traits can vary independently of each other through the year, facilitating the flexible response of physiological measures to environmental conditions. This pattern can further explain the reduced within-individual consistency in physiological measures we found.

To conclude, we found that coccidian parasites have a minor to no effect on annual variation in immune function and markers of antioxidant status in captive house sparrows. The absence of a relationship or an inconsistent correlation between coccidian infestation and physiological measures of male and female birds belonging to infestation and medication groups support our finding of a lack of any effect of these parasites on the circannual rhythm of physiological traits. Future studies that combine the advantages of experimental manipulations in captive and wild-living birds and that use parasites with more severe effects on hosts will be important for better understanding parasite-mediated changes in physiological traits over the annual cycle.

Acknowledgments

K. Sándor, A. Marton, G. Osváth, J. Veres-Szászka, and L. Bărbos kindly helped us during the fieldwork or measurement sessions. Adela Pintea and Dumitria Rugina kindly provided help with malondialdehyde, total antioxidant status, and uric acid assays. The research of P.L.P. was supported by TÁMOP-4.2.4.A/2-11/1-2012-0001 "National Excellence Program," and that of C.I.V. was supported by TÁMOP-4.2.2/B-10/1-2010-0024. These projects were sponsored by the European Union and by Hungary, cofinanced by the European Social Fund. The research infrastructure was supported by a research grant (PN II. RU TE 291/2010) of the Romanian Ministry of Education and Research. The experimental procedures were licensed by the Romanian Academy of Sciences (license 2257).

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