

Time of day, age and feeding habits influence coccidian oocyst shedding in wild passerines

Guillermo López *, Jordi Figuerola, Ramón Soriguer

Dto. Biología Aplicada, Estación Biológica de Doñana, Avda. María Luisa s/n, 41013 Seville, Spain

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Abstract

Protozoan coccidia are one of the most common intestinal parasites in birds. Ordinary coccidian detection and quantification techniques have proved to be inaccurate for wild passerines due to the existence of marked oocyst shedding rhythms throughout the day. Previous studies have suggested that these rhythms should be taken into account when analysing coccidian load and prevalence data, but their pattern and magnitude still remain poorly known. In this study we characterised shedding rhythms in the field by means of 406 samples of faeces taken from two species of passerines with different diets: the European Serin (a granivorous species), and the Garden Warbler (an insectivorous species). Both coccidian prevalence and load were two-phased, with maximums occurring in the afternoon. Oocyst elimination remained consistently high during the second half of the day, whereas prevalence peaked during the afternoon, lowering throughout the evening. This pattern was found in both species. We found a high repeatability of prevalence and intensity when differences between the morning and afternoon were statistically controlled. As a result, we suggest that sampling periods used in the analysis of coccidian prevalence and/or load studies should take into account these differences in times of shedding and be limited to the afternoon, otherwise a statistical control of this factor will be required.

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1. Introduction

Parasitism is widespread in nature and its importance in the ecology and evolution of organisms is well known (Thomas et al., 2005). Host-parasite relationships in avian passerine species have become a common focus of research over the last decade. Many studies have centred on ecto- and blood parasites, whereas due to the lack of an accurate quantification method, rather fewer have focused on endo-parasites. Coccidian protozoa are intestinal parasites that are found in most vertebrate species and which have been shown to be involved in many ecological avian processes (McGraw and Hill, 2000; Hill, 2002). Most (families *Eimeriidae* and *Cryptosporidiidae*) are monoxenous, the transmission between individuals taking place via infective

oocysts released in faeces. The only non-invasive method of determining the presence and burden of these coccidians is to detect and count oocysts in host faeces (Watve and Sukumar, 1995). This is, however, an inaccurate method for field studies, in which only one sample can usually be taken at a time, because circadian variation in oocyst shedding has been observed in many species. For example, variation is known to occur in some species of the genus *Eimeria* that infect domestic chickens and partridges (Clarke, 1979; Williams, 1995; Villanúa et al., 2006). Passerines are mainly infected by species belonging to the genus *Isospora* (reviewed by Giacomo et al., 1997; McGraw and Hill, 2000), in which a host-dependent circadian variation in oocyst shedding has also been observed (Boughton, 1933). Although previous studies with passerines have suggested that oocyst discharge is much greater in the afternoon than in the morning, knowledge of this process is still deficient and indeed many of these studies have only

* Corresponding author. Tel.: +34 954232340x154; fax: +34 954621125.
E-mail address: guillermo@ebd.csic.es (G. López).

focused on prevalence (Brawner and Hill, 1999; Hudman et al., 2000; Brown et al., 2001). Other studies have assumed, but not tested, the existence of alternative morning/afternoon states (Schwalbach, 1961; Hudman et al., 2000; Misof, 2005). Eurasian Blackbirds (*Turdus merula*) and their gastrointestinal parasites: A role for parasites in life-history decisions? ULB, Bonn.), or have been performed on birds kept in captivity for days or weeks at a time (Boughton, 1933; Brawner and Hill, 1999; Dolnik, 1999a). Moreover, the effect that diet and the natural activity rhythms of hosts may have on oocyst shedding rhythms has never been analysed. Since digestive physiology varies with feeding habits (Sturkie, 1986), oocyst shedding may well also be influenced by these variables. Thus, knowledge of patterns occurring in circadian rhythms with different feeding habits will help researchers to collect, analyse, and interpret data correctly. In this paper, we describe coccidian oocyst shedding rhythms in the field over the whole day in two species of passerines with different feeding patterns. Our goal was to achieve an accurate coccidian detection and quantification method in order to establish the best sampling period to use in field studies. We analysed oocyst presence and burden in faeces of both a seed-eater and an insectivorous species of free living passerines.

2. Materials and methods

2.1. Field work

Because of their abundance and diet specificity, European Serins (*Serinus serinus*, Linnaeus 1766) and Garden Warblers (*Sylvia borin*, Boddaert 1783) were chosen as models of seed-eater and insectivorous birds, respectively. Birds were trapped during daylight between March and May in 2004 and 2005 in a tree nursery in the city of Seville (37°23'11"N, 5°57'46"W), with mist nets placed amongst bushes. Birds were individually marked with numbered aluminium rings, sexed and aged (as juveniles or adult birds) according to Svensson (1996). Capture and handle of the birds were carried out with animal ethics approval by the Spanish Environment Ministry. Whereas *S. borin* is strictly migrant in the study area, *S. serinus* is a common breeder. Consequently, juveniles were only present in the samples of *S. serinus*. Birds were kept individually in cloth bags for 20 min to collect faecal samples and were then released. Of the total trapped birds 49.9% of *S. serinus* and 84.2% of *S. borin* produced faeces during the 20-min capture period. Between 0.5 and 1 mg of faeces were placed in individually marked vials containing 5% formol and the collection time was recorded for each sample. Because urine does not contain oocysts and given that its mass would have affected the sample mass, we only analysed the intestinal component of the dropping and rejected the part corresponding to urine. When the two fractions could not be separated, the sample was excluded from the study. A total of 406 samples (252 from *S. serinus* and 154 from *S. borin*) were included in the analysis.

2.2. Laboratory method

Samples were filtered through a double piece of cotton-lint cheesecloth (which oocysts easily pass through) and then homogenized to obtain a dilution. This was scanned for coccidian oocysts in a McMaster chamber (Williams, 1973). This method of quantification is the most widely used method in passerine coccidia research (Hörak et al., 2004; Misof, 2004; Hörak et al., 2006). Since the low concentration of faecal debris made it easy to find oocysts, no oocyst concentration method (such as flotation) was used, in order not to affect oocyst density. The scanning area of the McMaster chamber contains 300 µl of sample, and it is divided by nine parallel lines into 10 rectangular sections. Two scanning areas were examined for each sample. Subsequently, 200 µl of the same dilution was taken from the chamber and dried out in a 54 °C heater; the extract was then weighed to the nearest 0.0001 g in an Ohaus Voyager precision weight (Ohaus, Switzerland). Coccidian load values (expressed as the number of oocysts per milligrams of dry extract of faeces) were obtained by dividing the number of oocysts counted in the chamber by the estimated mass of the scanned sample. Prevalence was calculated as the percentage of individuals releasing oocysts in faeces out of the whole group of sampled birds. Unlike chicken *Eimeria* oocysts found in faeces, most of the oocysts in our sample were already sporulated, allowing identification to genus level. Based on size and number of sporocysts, oocysts were identified as *Isospora*-like. The repeatability of coccidian load values was estimated by blindly counting the load of 10 individuals twice and calculating the intra-class correlation (Lessels and Boag, 1987). Repeatability of coccidian load estimates from the same samples was very high (97%). This gave confidence to the accuracy of oocysts counts.

2.3. Statistical analysis

Because daylight length varied by 2 h and 36 min during the sampling period, hourly data from different days were not comparable. To obtain comparable hourly data, this variation was controlled by comparing the hourly data to total day length. The time of sample collection was transformed in the following way: relative hour = (time of sample collection – time of daybreak)/(time of sunset – time of daybreak). Values for the relative hour ranged from 0 to 1. As coccidian load and prevalence does not necessarily change linearly with time, relative hourly values were standardized by rounding off to the next decimal point. Thus, the standardized hour was analysed as a 10-level factor. Coccidian prevalence and load data did not fit a normal distribution, so traditional parametric statistical methods based on variance analysis could not be performed. Generalized linear models (GLMs) were performed instead. GLMs allow a more versatile analysis of correlation than standard methods, since the error distribution of the dependent variable

and the function linking predictors can be adjusted to the characteristics of the data.

The effects of standardized hour, species and year on coccidian load and prevalence were analysed as independent factors. To test for differences between species in the hourly patterns of oocyst shedding, the interaction between the standardized hour and species was included in the initial models. The coccidian load was analysed with a negative binomial distributed error and a log link, while prevalence was analysed by means of a binomial distributed error and a logit link. Models were fitted using the GENMOD procedure with the III type of sum of squares and backwards stepwise selection procedure using the SAS 9.1 statistical package (Littell, R.C., Milliken, G.A., Stroup, W.W., Wolfinger, R.D., 1996 SAS System for Mixed Models. SAS Institute Inc. Cary, NC, USA). The least significant variable was excluded from the model and the model was refitted to the data until all of the remaining variables contributed significantly to the fit (as judged by partial P -values < 0.05). To test the effect of age on oocyst shedding patterns, a separate analysis using only data for *S. serinus* was carried out, since no yearlings of *S. borin* were captured. In this analysis, the standardized hour, year, age (expressed as yearling/adult) and the age*hour interaction were included as factors.

Because preliminary analysis suggested the existence of a morning/afternoon dichotomy regarding load and prevalence, the repeatability of both batches of data was calculated with and without taking into account the morning/afternoon factor (defined as the first and second half of the day). For these analyses, 48 samples of 23 individuals (nine *S. borin* and 14 *S. serinus*), captured at different hours and on different days, were used. A random factor controlling for individuals was included in the model using the GLIMMIX procedure in SAS 9.1. The repeatability of prevalence data was calculated using the latent variable approach described by Browne et al. (2005). We are not aware of a similar approach for variance decomposition and repeatability calculation for variables with a negative binomial distribution and so the repeatability of the coccidian load data was calculated with log transformed values and a normal distribution error. Given that 14 individuals were captured both in the morning and the afternoon on different days (10 *S. serinus* and four *S. borin*) we used a

Wilcoxon non-parametric test to compare the coccidian load during these two periods.

3. Results

3.1. Effects of hour, species and age on coccidian prevalence

Standardized hour was the only variable significantly related to coccidian prevalence in our model (Table 1). No significant differences in prevalence were found either between species or between yearling and adult *S. serinus* ($\chi^2 = 0.21$, d.f. = 1, $P = 0.649$). The test of mean differences in the minimum squares showed that prevalence was similar and peaked in periods 6, 7, 8 and 9. This means that coccidian elimination in infected individuals takes place mainly between 1/2 and 9/10 of the daylight period (Fig. 1). The repeatability of coccidian prevalence was low when morning/afternoon was not taken into account (5.26%), but when this factor was controlled for, the repeatability was very high (90.19%).

3.2. Effects of hour, species and age on coccidian load

Both standardized hour and species, as well as the interaction between these two factors, were significantly related to coccidian load (Table 1). No differences in coccidian load were found between years. The test of differences in minimum squares identified two homogeneous periods in the day in both species (standardized hour periods 1, 2, 3, 4 in one group, and 6, 7, 8, 9 in the other group; neither of the groups was significantly different from each other in hour period 5). This result shows that oocyst elimination was homogeneously low during the first 2/5 day and homogeneously high during the second half of the day (Fig. 2). Lower oocyst discharge occurred in *S. borin* (minimum squares mean \pm standard error in log scale: 4.29 ± 0.21 ; $n = 49$) than in *S. serinus* (4.49 ± 0.15 ; $n = 109$). The test of differences in minimum squares showed that this difference was limited to period 2 ($\chi^2 = 4.27$, d.f. = 1, $P = 0.04$) and to the afternoon periods 6 ($\chi^2 = 75.22$, d.f. = 1, $P < 0.0001$), 7 ($\chi^2 = 9.54$, d.f. = 1, $P = 0.002$), and 8 ($\chi^2 = 4.21$, d.f. = 1, $P = 0.04$). In *S. serinus*, the oocyst load was greater in yearlings (5.38 ± 0.24 , $n = 64$) than in adults (4.48 ± 0.21 , $n = 41$; $\chi^2 = 7.66$, d.f. = 1, $P = 0.005$). Twelve out of 14 individuals trapped both in

Table 1

Models analysing the effects of year, standardized hour and species, as well as their interactions, for both coccidian prevalence and load

Factor	Prevalence				Load			
	Estimate	F	d.f.	P	Estimate	F	d.f.	P
Standardized hour	−2.525 to 1.704	142.36	9	<0.0001	−2.591 to 3.374	78.68	8	<0.0001
Species		0.08	1	0.772	0.658	5.66	1	0.017
Year		0.17	1	0.683		2.51	1	0.113
Standardized hour*Species		9.86	8	0.277	−5.357 to 1.432	43.56	8	<0.0001

Model selection followed a backwards stepwise selection procedure. For variables included in the final model, parameter estimates and statistical significance are given. For variables not included in the final model the statistical significance when added to the final model is given.

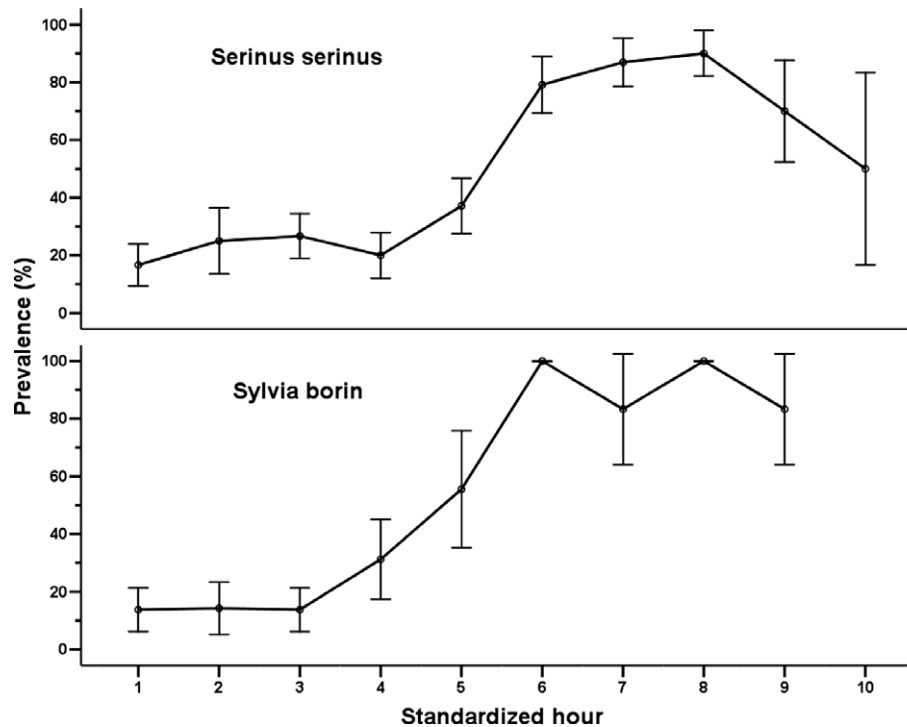


Fig. 1. Estimated coccidian prevalence (expressed as the percentage of individuals releasing oocysts in faeces) throughout the daylight period showed higher values during the second half of the day. Lines represent the S.E.M. for each period.

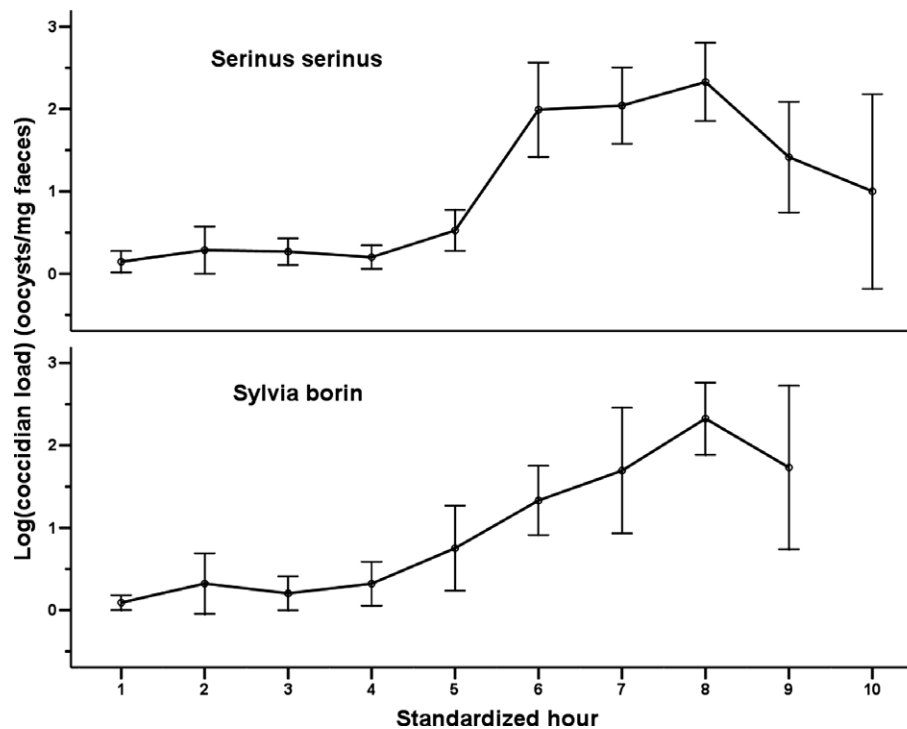


Fig. 2. Estimated coccidian load (expressed as number of oocysts released in each milligram of dry extract of faeces) throughout the daylight period showed a peak during the late afternoon. Mean \pm standard error of log transformed ($\log(\text{value} + 1)$) load values are represented.

the morning and the afternoon showed higher oocyst discharges during the afternoon; in the other two birds no shedding occurred in either of the periods (Wilcoxon Test,

$Z = -3.59$, $P = 0.002$; Fig. 3). Coccidian load estimates were only repeatable after controlling for the morning/afternoon factor (0% without control, 41.92% after

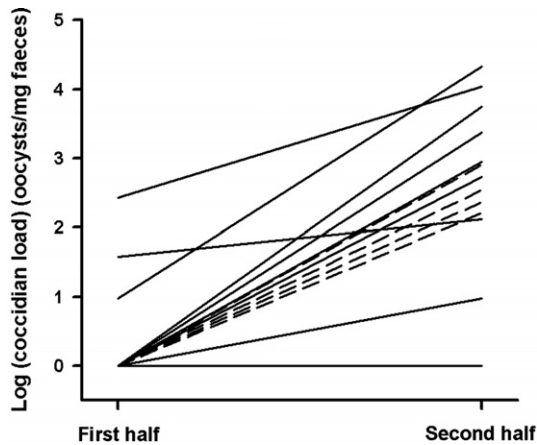


Fig. 3. Differences in coccidian load between morning and afternoon for 14 individuals sampled in both periods. Load values were higher during the afternoon for all individuals except for two, which presented no oocyst shedding in either period (and so their lines overlap). Solid lines represent *Serinus serinus* ($n = 10$) and dashed lines *Sylvia borin* ($n = 4$). Coccidian load data are presented log transformed ($\log(\text{value} + 1)$) in order to get a better idea of the differences between individuals.

controlling for the factor). Since recaptures were usually separated by several days (range 0–66 days), it is possible that oocyst loads had changed during this time. For this reason much higher repeatability estimates were found when using data from individuals recaptured within fewer than 6 days (76.35%, controlling for morning/afternoon).

4. Discussion

This research shows that coccidian oocyst shedding in free-living passerines presents clear circadian rhythms, which strongly affect the estimates of both coccidian prevalence and load based on oocyst-counting in chamber. In the wild, these findings are not affected by changes in diet or in the typical activity patterns of birds kept in captivity. We believe this study is the first report of the existence of differences in coccidian load between species with different feeding habits. It also suggests that coccidian load is related to age in wild passerines. The results indicate that prevalence is not related to bird age, a finding that agrees with previous work (Dolnik, 1999a; Hudman et al., 2000; Misof, 2005). Yearling *S. serinus* exhibited greater oocyst discharge in their faeces than adults. This is to be expected given immune system physiology, since the presence of coccidia leads to the acquisition of immunity (Rose and Hesketh, 1982; Lillehoj and Trout, 1996) and so hosts will increase their immune response in cases of consecutive exposure to parasites (Guzman et al., 2003; Ding et al., 2004). This would explain the drop in coccidian load as birds age. Both coccidian load estimates and prevalence estimates revealed circadian bimodal cycles with oocyst discharge peaks in the afternoon. These patterns agree with previous results obtained from captive populations (Dolnik, 1999a; Brawner et al., 2000). The two species studied did not differ with respect to prevalence estimates, but

did with respect to load estimates, a fact that indicates that different physiologies possess different oocyst elimination patterns. This difference was not caused by the presence of yearling *S. serinus* in our sample because excluding yearlings from our model did not change the results (results not shown). It is known that coccidian oocyst shedding is controlled by host physiology (Boughton, 1933; Dolnik, 1999a,b), and the physiological differences between feeding habits is probably responsible for this difference. The mechanisms underlying this process, however, are still unclear. A variation in the amount of faeces produced according to time of day could influence these rhythms. Nevertheless, researchers must be aware that highly reliable estimates of both prevalence and load can be obtained if, and only if, time of day is taken into account. Estimates of prevalence and load made without considering time of day were not repeatable, but were highly repeatable when a factor coding for morning/afternoon was included. Another good method for obtaining accurate data seems to be to restrict the sampling period. Coccidian load sampling should be restricted to the second half of the total daylight time, whereas in the case of prevalence, samples should be taken at between 1/2 and 4/5 of the daylight time. This more restrictive period should thus be considered as the best period for obtaining reliable data for both coccidian prevalence and load because even heavily infected individuals could not release oocysts during the morning.

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