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PARASITE SHEDDING IS HIGHLY INFLUENCED BY AGE, TIME OF DAY, AND SAMPLING DATE IN SPOTLESS STARLING *STURNUS UNICOLOR* NESTLINGS

LA EXCRECIÓN DE PARÁSITOS SE ENCUENTRA ALTAMENTE INFLUENCIADA POR LA EDAD, LA HORA DEL DÍA, Y LA FECHA DE MUESTREO EN POLLUELOS DE ESTORNINO NEGRO *STURNUS UNICOLOR*

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SUMMARY.—Investigating the influence of parasitic infection on animals requires precise estimates of the infection status of individuals. In the case of intestinal parasites, infection condition is often indirectly determined by the presence and abundance of parasite propagules in faeces. However, parasite shedding is affected by ontogenetic, daily and seasonal patterns that are rarely identified, particularly for nestlings of wild passerines. We investigated the effect of age and time of day on coccidial oocyst shedding in Spotless Starling *Sturnus unicolor* nestlings and the variation over the breeding season in the prevalence and intensity of coccidial infection. Our study demonstrated that coccidial oocysts can be detected in nestling faeces as early as seven to ten days after hatching but not before. Late afternoon, when prevalence was 98% of samples, was the most likely time to detect coccidial oocysts, compared with a prevalence of 43% in early morning samples. Infection intensity in starling nestlings also increased throughout the day, averaging (± S.E.) 102 ± 27 oocysts per gram of faeces in the early morning to 29,570 ± 7,533 oocysts per gram at the end of the day. Lastly, we observed an increase in infection intensity, in end of day samples, from a mean (± S.E.) of 27 ± 14 oocysts per gram of faeces at the beginning of the season to 27006 ± 5116 oocysts per gram. Also, we found an increase in prevalence of coccidial infection as the season progressed: from 58% at the beginning of the season to 87% at the end. This article provides useful methodological information for future studies

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of nestlings in free-living avian species. We also report some considerations that may help to establish appropriate protocols to investigate the influence of this type of parasitic infection on different aspects of bird biology, minimising biases and errors.—De la Peña, E., Muriel, J., Gil, D. & Pérez-Rodríguez, L. (2024). Parasite shedding is highly influenced by age, time of day, and sampling date in Spotless Starling *Sturnus unicolor* nestlings. *Ardeola*, 71: 307-320.

Key words: circadian pattern, coccidia, development, faeces, infection, spotless starling.

RESUMEN.—La investigación de la influencia de la infección parasitaria en los animales requiere estimaciones precisas del estado de infección de los individuos. En el caso de los parásitos intestinales, el estado de infección suele determinarse indirectamente por la presencia y abundancia de propágulos del parásito en las heces. Sin embargo, la excreción de parásitos se ve afectada por patrones ontogenéticos, diarios y estacionales que rara vez se identifican, especialmente en el caso de los polluelos de paseriformes silvestres. En este trabajo investigamos el efecto de la edad y la hora del día sobre la excreción de ooquistes de coccidios en polluelos de estornino negro *Sturnus unicolor,* así como la variación a lo largo de la época de reproducción en la prevalencia e intensidad de la infección por coccidios. Nuestro estudio demostró que los ooquistes de coccidios pueden detectarse en las heces de los polluelos entre siete y diez días después de la eclosión, pero no antes. El final de la tarde fue el momento donde la probabilidad de detectar ooquistes de coccidios (del 43% a primera hora de la mañana al 98% al final del día) y la intensidad de infección fue máxima (de media ± E.S. 102 ± 27 ooquistes por gramo de heces a primera hora de la mañana a 29.570 ± 7.533 ooquistes por gramo de heces al final del día). Por último, se observó un aumento de la intensidad (de media ± E.S. 27 ± 14 ooquistes por gramo de heces al principio de la temporada a 27006 ± 5116 ooquistes por gramo de heces al final de la época de reproducción) y prevalencia de la infección por coccidios a medida que avanzaba la temporada de cría (de un 58% al inicio de la estación a un 87% hacia el final de esta). Este artículo aporta información metodológica pertinente para futuros estudios sobre polluelos de especies de aves silvestres. Se exponen algunas consideraciones que pueden ayudar a establecer protocolos apropiados para investigar la influencia de este tipo de infección parasitaria sobre distintos aspectos de la biología de las aves, minimizando sesgos y errores.—De la Peña, E., Muriel, J., Gil, D. y Pérez-Rodríguez, L. (2024). La excreción de parásitos se encuentra altamente influenciada por la edad, la hora del día, y la fecha de muestreo en polluelos de estornino negro *Sturnus unicolor. Ardeola*, 71: 307-320.

Palabras clave: coccidios, desarrollo, estornino negro, heces, infección, patrón circadiano.

INTRODUCTION

The significance of parasites in the biology of their hosts lies in their impact on fitness. Parasites influence different key aspects of host ecology and behaviour, and these effects may range from the individual to the population level (Clayton & Moore, 1997; Wilson *et al*., 2019). Evidence suggests that parasites may constitute an important selection pressure on wild bird populations (Møller *et al*., 1990; Knowles *et al*., 2010). In these, parasites affect multiple fitness-related traits of hosts e.g. body condition, survival and breeding success, among others (Clayton & Moore, 1997; Wilson *et al*., 2019). Parasite load often varies according to host age, sex and environmental conditions (Hudman *et al*., 2000; Hayward *et al*., 2009; Frigerio *et al*., 2016). However, the ecology and impact of parasitism on birds in early life has been much less studied than in adulthood, and this bias limits our understanding of the implications of parasites throughout all life stages. Clearly, understanding the factors that determine the presence and abundance of parasites –not only among adults, but also during early life–, and identifying methodological sources

of error and sampling biases, are essential to obtaining reliable data for modelling host-parasite dynamics.

Coccidia (Protozoa, Apicomplexa) are common intracellular intestinal endoparasites in granivorous and omnivorous birds (Brawner & Hill, 1999; Dolnik, 1999a, b; Brown *et al*., 2001; Misof, 2004). During foraging, hosts ingest the sporulated oocysts, which are the environmentally resistant infective forms of this parasite. The endogenous stage consists of asexual and sexual multiplication of the parasite in the host gut (Hammond & Long, 1973). Non-sporulated oocysts derived from sexual reproduction are released into the environment via host faeces. The exogenous phase includes sporulation of the oocysts ready to be ingested by a new host (Malo, 2013). The coccidial prepatent period, i.e. the time between the parasite is ingested and the first appearance of oocysts in faeces, lasts around six days (Cordero del Campillo & Rojo-Vázquez, 2000), although this period may be influenced by environmental factors and host condition. Indeed, infection by coccidia may show seasonal variations, either because weather conditions affect the coccidial life cycle (e.g. high temperatures favour the sporulation phase; Merck *et al*., 2010; Malo, 2013), or because of seasonal variations in host exposure or susceptibility (Martin *et al*., 2008; Acevedo-Whitehouse & Duffus, 2009; Hawley & Altizer, 2011).

Previous studies have shown that host age also affects coccidial load: the intensity of coccidial infection decreases rapidly with host age in different passerine species (Dolnik, 1998; Dolnik, 2002), with high prevalence in nestlings and yearlings as compared to adults (Kruszewicz, 1995; Dolnik, 2002; López *et al*., 2007). However, our understanding of the ontogeny of coccidial infection during early life is still limited. Finally, coccidial oocyst shedding also shows within-day variation, oocyst abundance in faeces being greatest in the late afternoon in adults of several avian species, showing a clear circadian pattern (e.g. Villanúa *et al*., 2006; López *et al*., 2007; Dolnik *et al*., 2010; Knight *et al*., 2018). However, the dynamics of coccidial excretion in altricial nestlings have not been studied so far. There are reasons to predict that nestling excretion patterns may differ from adults. Firstly, nestlings inhabit in a much more stable environment, particularly in the case of hole-nesting species, which may potentially buffer daily physiological rhythms of both hosts and parasites. Secondly, early-stage nestlings have a relatively immature acquired immune system as compared to adults (e.g. Muriel *et al*., 2021). If parasite propagule shedding patterns result from an interaction with the hosts' immune function, these would differ between adults and nestlings.

The Spotless Starling *Sturnus unicolor* (herafter 'starling') is a medium-sized hole-nesting passerine that often lays two clutches per season (Veiga & Polo, 2016). In populations from areas of continental Mediterranean climate, characterised by hot and dry summers, nestlings from the second wave of clutches develop under harsher conditions than nestlings from earlier clutches, as temperature rises and food becomes scarcer as the season advances (Salaberria *et al*., 2014; Muriel *et al*., 2015). We conducted a non-invasive study of the shedding patterns of coccidial oocysts by starling nestlings to detect the age (in days) at which coccidial infection is first detectable in faeces and the ontogenetic changes in parasite infection. We also explored time of day variation in coccidial detectability in faeces, as well as changes in estimated infection intensity, to provide reliable and accurate guidelines for sampling this type of parasite in nestling passerines. Finally, we examined the coccidial excretion dynamics throughout the breeding season in nestlings of our study species.

MATERIAL AND METHODS

Study site and sample collection

This study was performed on a starling population in central Spain (Soto del Real, Madrid) that utilises 246 nest boxes that are monitored during the breeding season from March to July. In this area, most pairs lay two clutches: the first in early April and the second around the end of May (Salaberria *et al*., 2014; Muriel *et al*., 2015), which results in two brood cohorts. However, pairs that fail early in their first breeding attempt often lay replacement clutches, which results in a continuous occurrence of clutches between these two main laying periods. Starling nestlings fledge approximately at 22 days after hatching, and males are significantly larger than females after completing their linear growth phase of growth (Muriel *et al*., 2021).

To identify the age at which starling nestlings start to shed coccidial oocysts and the potential time of day variations, we sampled nestlings from the first brood cohort of the 2022 breeding season, between the 3rd May and the 16th of May. Faecal samples were collected from a total of 52 nestling starlings (27 females and 25 males) from 13 nests. To minimise the potential seasonal variations in this dataset, we selected for broods hatched within a two-day window. Faecal sacs from each individual were collected in Eppendorf tubes after removing the white urate region and kept refrigerated (4°C) until analysis, within two weeks after collection. Faeces were collected between 12:25 and 19:46 hrs (GMT + 1) on days 3, 6, 10, 14, and 16 post-hatching in all cases. All individuals were marked on day 3 by distinctive down trimmings for individual recognition, and with numbered aluminium rings from day six onwards, allowing a repeated measurements sampling schedule. On day six, a blood sample was taken from the brachial vein and stored in ethanol 100% for molecular sexing, using a previously established protocol (Griffiths *et al*., 1998).

When nestlings were 14 days old, the same broods were used to study time of day variations in oocyst shedding. Each nestling was sampled three times daily, within each of the following periods: 08:15-10:45, 12:25-17:02 and 18:25-20:30 hrs. These intervals were defined to ensure an even sampling distribution across the day, although for statistical analyses we considered the exact sampling hours (see below). Sunrise in our study area was at 07:00 hrs and sunset at 21:24 hrs during data and sample collection. Samples were stored as described above.

To study our first objective, the pattern of oocyst shedding with age, a total of 206 faecal samples were analysed. To address the second objective, differences in shedding pattern with time of day, a total of 151 faeces were analysed. 50 samples from this subset of data were also used to investigate the first objective due to the similarity with the time at which faeces were collected from the same individuals at other ages (between 12:25 and 17:02).

To assess whether oocyst shedding varies throughout the breeding season, we analysed data collected in 2010. In that case, samples were stored in 10% formaldehyde at 4°C until coprological analyses were performed (Muriel *et al*., 2017). For this objective, we collected samples from 252 starling nestlings (121 males and 131 females) from 75 broods raised across the breeding season (from 15th May to 6th July). Faeces were collected from individuals between 14 and 16 days of age (mean ± S.E.: 15.4 ± 0.92), and data were pooled for the analysis as we observed no significant differences among these age groups (age effect: $X^2 = 6.159$, d.f. = 1, p = 0.281). In 2010 the time of faecal sample collection ranged from 17:00-21:15 hrs, within the time of day of highest detectability of coccidial oocyst shedding

(see Results: "Time of day variation in coccidial oocyst detection").

For both sample periods (2010 and 2022), nestlings usually defecated as soon as they were picked up from the nest box. In the exceptional cases when this did not happen, they were left in a container for up to five minutes until they defecated.

All applicable EU and national guidelines for the care and use of animals were followed for this experiment. Procedures were approved by the Dirección General de Agricultura y Ganadería, Junta de Comunidades de Castilla-La Mancha (ref. 13-2022).

Coprological analyses

Faecal samples were analysed with a zinc sulphate-based centrifugal flotation procedure, as described by Villanúa *et al*. (2006) and Muriel *et al*. (2017). This quantitative analysis of coccidial oocysts was carried out within 15 days of collection in the field to ensure no sporulation and degradation of the oocysts. Faecal samples, equivalent to 0.2-0.3g of fresh faeces were homogenised and weighed (accuracy = 0.001g). In the case of 2010 samples, the same amount of faecal material was extracted from the tubes and spread on filter paper for five minutes to remove formaldehyde remnants (Muriel *et al*., 2017), and then weighed as before. All samples, irrespective of storage medium, were suspended in 5ml of a saturated zinc sulphate solution by vigorous mixing in a porcelain mortar, filtered and transferred to a MacMaster chamber for oocyst counting. To calculate their concentration in terms of oocysts per gram of faeces we considered exact sample weight analysed and dilution. A random subset of 17 samples collected across the 2010 breeding season was sporulated in the laboratory in an aqueous solution of potassium dichromate and inspected under the microscope, allowing us to confirm that oocysts were *Isospora* sp. We performed the coprological analysis on 93% of the three-day-old samples and 24% of the six-day-old samples collected in 2022, and on all samples collected in both 2010 and 2022 from ten-day-old and older nestlings.

Statistical analyses

For our coprological data, we defined coccidial *prevalence* as the non-parasitised (0) or parasitised (1) status of an individual, and the *intensity of infection* as the number of coccidial oocysts counted per gram of faeces from an infected individual (i.e. whose prevalence = 1) (Margolis *et al*., 1982; Bush *et al*., 1997).

Our dataset from coccidial oocyst counts had a large proportion of zero values. Thus, to explore how both the prevalence and the intensity of infection varied with nestling age, time of day, and during the breeding season, we used the *glmmTMB* package (Brooks *et al*., 2017) to build hurdle generalized linear mixed models fitting to a truncated negative binomial error distribution. Hurdle models split into a binary process which analyses the parasite prevalence (containing zero values, zero-inflation model), and a counting process by which the intensity of infection can be assessed when it has occurred (containing positive counts, conditional model). To test the effect of age on coccidial infection status, we included 'sex' and 'nestling age' as fixed terms. For 14 days of age (for which we had the infection status at three different times of the day for each individual) we used the data from faeces collected during the time frame closest to the collection time frame for the other ages (between 15:00-19:46 hrs). To examine time of day variations in coccidial shedding at 14 days old, we built a second model with 'sex' and 'sampling time' as fixed terms. For sampling time, we considered the exact time of sample collection

using sunrise as the reference time (07:00 hrs, GMT + 1, is time = 0). In both models, as random term we included individual identity nested in its own nest. The reference level for sex was the female. To explore the effect of breeding season stage (i.e., date) on coccidial infection, we employed another model using coccidial counts from 2010 samples and considering the exact nestling age, sampling time and Julian date (1 = January 1st) when the faeces were collected as fixed factors. Nest was included as a random effect.

We checked the normal distribution of the model residuals using a Shapiro-Wilks test and we assessed the assumptions of homogeneity of variance by plotting residual versus fitted values. We examined the presence of outliers and potential influential data points using Cook's distance graphs. To avoid multicollinearity between variables, we calculated the variance inflation factors (VIFs; Alin, 2010) of each built model using the *usdm* package (Babak, 2015). In all cases, we found no evidence of collinearity (VIF < 1.00). All analyses were performed in R language v. 3.6.1 (R Core Team, 2020). The level of statistical significance was $p < 0.05$. Predictions were visualised with *ggeffects* (Lüdecke, 2018) and *ggplot2* (v3.1.1) was used for graphics (Wickham, 2016).

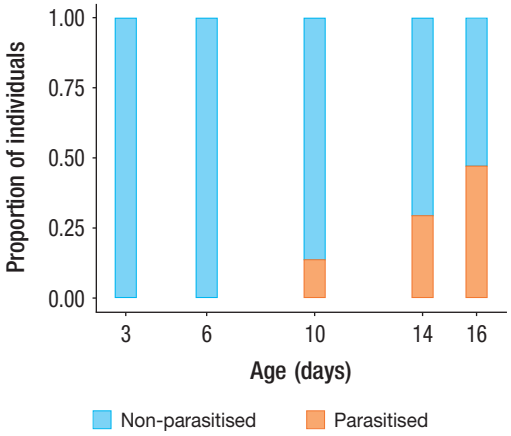

FIG. 1.—Age-related variations in coccidial prevalence in Spotless Starling nestling faeces. Columns indicate the proportion of individuals sampled in which coccidial oocysts were detected (orange, "parasitised") or non-detected (blue, "non-parasitised").
[Variaciones relacionadas con la edad en la prevalencia de coccidios de polluelos de estornino negro. Las columnas indican la proporción de individuos muestreados en los que oocistes de coccidios fueron detectados (naranja, "parasitado") o no detectados (azules, "no parasitados").]

RESULTS

Considering all samples collected in 2010 and only those from 14-day-old nestlings in 2022 (from the maximum detectability of shedding time of day), we found a prevalence of *Isospora* oocysts in starling nestling faeces of 81% (206/252) in 2010 and 98% (48/49) in 2022. Infection intensity was overdispersed in both datasets. In 2010, the intensity of infection ranged from 0.081 to 675,732 oocysts per gram of faeces (mean ± S.E. = 12,657 ± 3,320), whereas for 14-day-old nestlings sampled in 2022 the intensity of infection ranged from 82.918 to 165,476 oocysts/gram (mean ± S.E. = 27,007 ± 5,116).

Age-related variation in coccidia oocyst detection

Coccidial oocysts were not detected in any of the three- and six-day-old nestling samples analysed (Figure 1). Positive samples were only detected from ten-day-old (prevalence 13.7%) or older nestlings, and this prevalence gradually increased up to 53% in 16-day-old nestlings (Figure 1). This age-dependent increase in the prevalence of coccidia was statistically significant (Table 1a). No difference in coccidial prevalence was

TABLE 1

Results of the hurdle generalized mixed models testing for the effect of (a) nestling age, (b) sampling hour and (c) the sampling date on coccidial oocyst counts in Spotless Starling nestling faeces. In zero-inflation models, negative coefficients indicate higher prevalence. For the qualitative variable "Sex" the reference group for estimates was "female". The table also shows the variance and standard deviance (S.D.) of the random effects for each model.

[Resultados de los modelos mixtos generalizados hurdle para evaluar el efecto de (a) la edad de los polluelos, (b) la hora de muestreo y (c) la fecha de muestreo en los conteos de ooquistes en coccidios de heces de polluelos de estornino negro. En los modelos inflados de ceros, los coeficientes negativos indican alta prevalencia. Para la variable cualitativa "Sexo" el grupo de referencia de los estimadores fue "hembra". La tabla también muestra la varianza y desviación estándar (D.E.) de los efectos aleatorios de cada modelo.]

Term	Estimate ± S.E.	*Z*-value	*p*-value
a) Age			
Zero-inflated model			
Intercept	2.018 ± 0.392		
Age	−1.712 ± 0.356	−4.809	**<0.001**
Sex (female)	−0.138 ± 0.402	−0.342	0.732
Random factor (Variance ± S.D.):	*Individual*/*Nest*: 1.537e-08 ± 0.0001; *Nest*: 2.735e-01 ± 0.523		
Conditional model			
Intercept	7.388 ± 0.594		
Age	−0.205 ± 0.548	−0.374	0.708
Sex (female)	−0.228 ± 0.589	−0.387	0.699
Random factor (Variance ± S.D.):	*Individual*/*Nest*: 1.632e+00 ± 1.277e+00; *Nest*: 1.031e-24 ± 1.015e-12		
b) Time of day			
Zero-inflated model			
Intercept	−0.188 ± 0.312		
Time	−1.091 ± 0.213	−5.128	**<0.001**
Sex (female)	−0.394 ± 0.401	−0.982	0.326
Random factor (Variance ± S.D.):	*Individual*/*Nest*: 1.884e-8 ± 0.0001; *Nest*: 2.384e-1 ± 0.488		
Conditional model			
Intercept	7.624 ± 0.237		
Time	1.991 ± 0.136	14.570	**<0.001**
Sex (female)	0.053 ± 0.312	0.190	0.849
Random factor (Variance ± S.D.):	*Individual*/*Nest*: 1.663e-9 ± 4.077e-5; *Nest*: 3.278 e-11 ± 5.725e-6		

TABLE 1 (cont.)

Term	Estimate ± S.E.	*Z*-value	*p*-value
c) Sampling date			
Zero-inflated model			
Intercept	−3.108 ± 3.506		
Julian day	−0.900 ± 0.198	−4.556	**<0.001**
Age	0.096 ± 0.227	0.421	0.674
Sex (female)	0.304 ± 0.329	0.918	0.359
Conditional model			
Intercept	−1.283 ± 4.410		
Julian day	2.741 ± 0.278	9.887	**<0.001**
Age	0.507 ± 0286	1.773	0.076
Sex (female)	−0.411 ± 0.331	−1.124	0.215
Random factor (Variance ± S.D.): *Nest*: 2.506 ± 1.583			

detected between males and females. We did not find an effect of age or sex on the intensity of coccidial infection (Table 1a).

Time of day variation in coccidial oocyst detection

The hour of sample collection had a significant effect on the probability of detecting oocysts in 14-day-old nestlings, thus affecting the prevalence inferred (Table 1b; Figure 2a). Apparent prevalence ranged from 43% early in the morning, from 08:15 to 10:45 hrs., GMT + 1, (i.e. from 1 h 15 min to 3 h 45 min after sunrise in our study zone) to 98% in late afternoon, from 18:30 to 20:30, GMT + 1, (i.e. from 2 h 56 min to 1 h 6 min before sunset). Thus, 48 out of 49 of the faecal samples collected after 18:30 hrs tested positive for coccidial oocysts, even though many of these individuals (56.86%) did not shed oocysts when sampled earlier in the same day, before 10:45 hrs. We did not find any effect of on the probability of detecting coccidial oocysts (Table 1b). Similarly, sampling hour but not sex had a positive effect on the intensity of coccidial infection detected (Table 1b; Figure 2b).

Variation in coccidial infection across the breeding season

Coccidial prevalence significantly increased across the breeding season (Table 1c; Figure 3a). In first broods, 58% of nestlings tested positive for coccidial oocysts while at the end of the season, 87% of nestlings were infected. Given the slight degree of variation in sampling age in this dataset (mean ± S.E.: 15.443 ± 0.924, range: 14-18 days old), it is unsurprising that age did not exert a significant effect. No significant differences were found in coccidial prevalence between males and females (Table 1c). Likewise, coc-

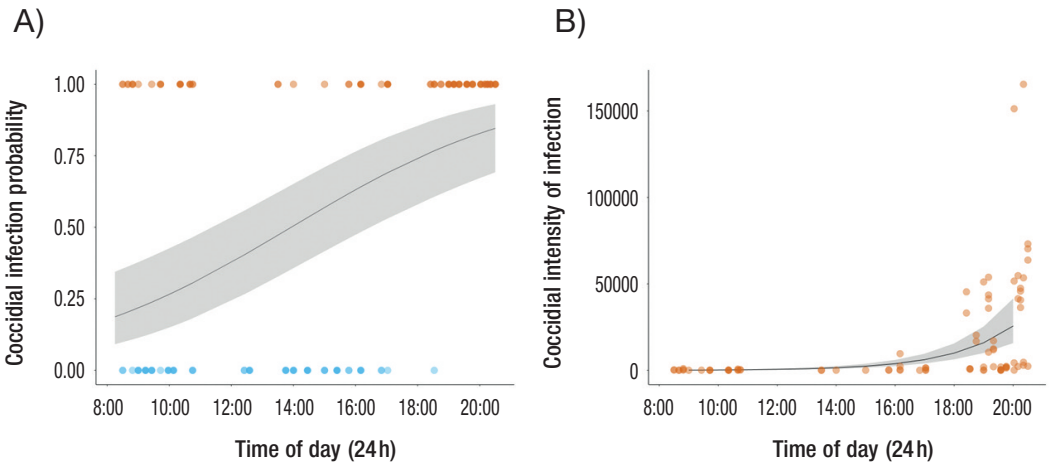

FIG. 2.—Correlation between sampling hour (24h) and (A) prevalence and (B) intensity of coccidial infection in faeces of 14-day-old Spotless Starling nestlings. During sample collection in the study area, the sunrise was 07:00 hrs and sunset time was 21:24 hrs (GMT+1). The continuous line represents the predicted values on both (A) the zero-inflation component (coccidial infection probability) and (B) the conditional component (coccidial intensity infection) of the hurdle mixed model (Table 1b). 95% C.I. marked in grey. Orange dots: coccidia detected; blue dots: coccidia non-detected. The variable colour intensity of dots results from data overlap.

[Correlación entre la hora de muestreo (24h) y (A) la prevalencia y (B) la intensidad de infección por coccidios detectada en heces de polluelos de estornino negro de 14 días de edad. Durante la recogida de muestras en la zona de estudio, el amanecer fue a las 07:00 hrs y el atardecer a las 21:24 hrs (GMT + 1). La línea continua representa los valores predichos para (A) el componente inflado de ceros (probabilidad de infección por coccidios) y (B) el componente condicional (intensidad de infección por coccidios) del modelo mixto hurdle (Tabla 1b). El intervalo de confianza del 95% aparece marcado en gris. Puntos naranjas: coccidios detectados; puntos azules: coccidios no detectados. La intensidad de color de los puntos se debe a la superposición de puntos.]

cidial infection intensity increased during the course of the breeding season, but was neither affected by nestling sex nor by the slight differences in nestling age at sampling (Table 1c, Figure 3b).

DISCUSSION

To our knowledge, this study is the first to show the variation in coccidial oocyst shedding in relation to age, time of day and breeding season stage in nestlings of a wild altricial bird species. We found that (1) coccidial oocysts could be detected on nestling faeces as early as seven to ten days-old, but not before; (2) there were strong variations in parasite shedding detection during the host's circadian cycle, late afternoon being the time of highest probability for detecting coccidial oocysts in the faeces and also showing the highest intensities of infection; and (3) there was an increase in both the prevalence and the intensity of infection as the breeding season progressed.

Previous studies on birds have shown age-related differences in the intensity (Dolnik, 1999a, b; Hudman *et al*., 2000) and time of day variation (López *et al*., 2007) of coccidial infection in adults. However, no prior

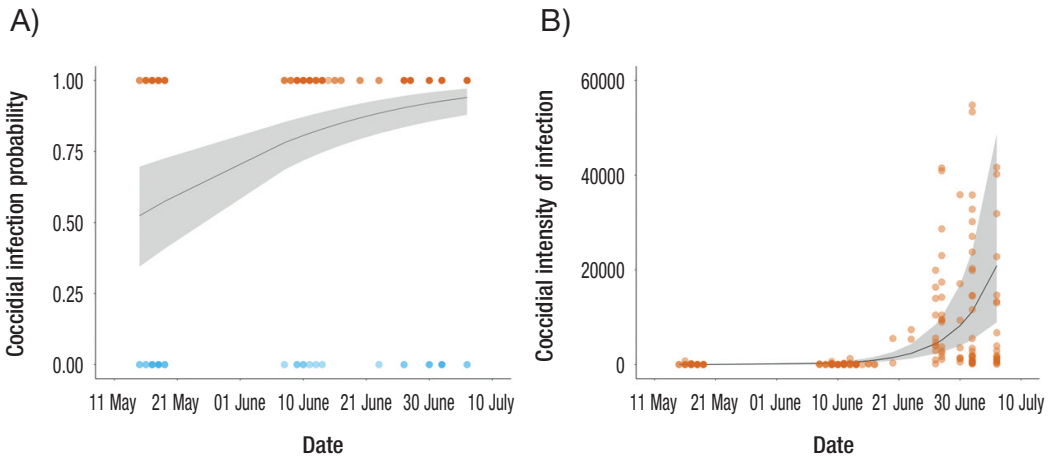

FIG. 3.—Correlation between sampling date across the breeding season and (A) prevalence and (B) intensity of coccidial infection in faeces of Spotless Starling nestlings. The continuous line represents the predicted values on both (A) the zero-inflation component (coccidial infection probability) and (B) the conditional component of the hurdle mixed model (Table 1c). 95% C.I. marked in grey. Orange dots: coccidia detected; blue dots: coccidia non-detected. The variable colour intensity of dots results from data overlap.

[Correlación entre la fecha de muestreo a lo largo de la época de reproducción y (A) la prevalencia y (B) la intensidad de infección por coccidios detectada en heces de polluelos de estornino negro. La línea continua representa los valores predichos para (A) el componente inflado de ceros (probabilidad de infección por coccidios) y (B) el componente condicional (intensidad de infección por coccidios) del modelo mixto hurdle (Tabla 1c). El intervalo de confianza del 95% aparece marcado en gris. Puntos naranjas: coccidios detectados; puntos azules: coccidios no detectados. La intensidad de color de los puntos se debe a la superposición de puntos.]

study has examined differences in coccidial shedding during the nestling stage, nor the age at which shedding begins. Our results indicate that the prepatent period for coccidia in starling nestlings is between seven and ten days after hatching. Given the biological cycle of coccidia, a certain period is required from when sporulated oocysts are ingested until they reach the intestine, reproduce first asexually and then sexually, and are finally shed in faeces as unsporulated oocysts (Hammond & Long, 1976). This result is consistent with previous research showing that coccidial prepatent periods in birds last between five and seven days (Cordero del Campillo & Rojo-Vázquez, 2000). It has previously been shown that lymphocytes in nestlings increase in number around nine days after hatching (Muriel *et al*., 2021), indicating that the acquired immune system may play a role when pathogens are present. Based on these previous studies, coccidia are unlikely to infect starling nestlings until this age. Therefore, triggering immune responses involving the acquired immune system is not required. Our results also reveal that from ten days after hatching, coccidial infection increases with nestling age. The prepatent period apart, nestlings must have a fully developed digestive system that enables the mechanical action of the gizzard and bile salts necessary oocyst excystation to occur (Doran & Far, 1965). Thus, host age may also affect this process, resulting

in lower infection rates in young nestlings and/or a longer prepatent period.

Coccidial shedding by starling nestlings revealed similar patterns of daily variation as those described for older birds (Brown *et al*., 2001; Misof, 2004). Peak shedding was identified in the late afternoon, in agreement with prior studies in adults across several avian species (Boughton, 1988; Brawner & Hill, 1999; Brown *et al*., 2001; Misof, 2004). This pattern appears to be ubiquitous and shared with all bird species regardless of latitude (Dolnik *et al*., 2011). This has been interpreted as an adaptive parasite strategy to avoid desiccation and ultraviolet radiation to which parasites are exposed ex-hosts (Martinaud *et al*., 2009). Previous studies have shown that the viability of coccidial oocysts decreases to almost half an hour when exposed to sunlight for one hour (Martinaud *et al*., 2009). Therefore, the daily pattern that we found corresponds with a characteristic of coccidia that enhances their probability of survival and transmission (Martinaud *et al*., 2009). Our results show that this trend is not affected by the fact that the hosts (nestlings of a hole-nesting passerine in this case) inhabit a relatively constant environment in which their daily activity rhythms seem mostly indirectly determined by paternal activities. For practical purposes, as when sampling adult individuals of avian species (Boughton, 1988; Brawner & Hill, 1999; Brown *et al*., 2001; Misof, 2004), when assessing the infection status of altricial nestlings, faeces should be collected in the late afternoon, at least three hours before sunset, to avoid biased infection estimates and minimise false negatives.

We found that coccidial prevalence and intensity in nestlings increased as the breeding season advanced. Two possible non-exclusive hypotheses may explain our results: (a) differences in nestlings' immune function and (b) the less favourable (to nestlings) environmental conditions later in the season that enhance the parasites' survival and host exposure. In our study population, as in most continental Mediterranean areas, avian breeding conditions worsen as the breeding season progresses due to higher temperatures and a sharp decrease in rainfall: in May 2020 mean daily temperature was 13.45°C and monthly rainfall was 1.547 l/m^2 but in July 2020 the equivalent figures were 25.46°C and 0.533 l/m^2 (data provided by the Spanish Meteorological Agency, AEMET). Heat stress has been shown to negatively impact nestling development in late broods of our study population (Salaberria *et al*., 2014), which grow in a harsher environment resulting in reduced nestling survival, condition at fledging, immunocompetence and recruitment prospects (Salaberria *et al*., 2014; Muriel *et al*., 2015; Gil *et al*., 2020; Redondo *et al*., 2022). Indeed, this marked seasonal decrease in fledgling quality is a ubiquitous pattern in most avian species and populations (Brinkhof *et al*., 1993; Verboven & Verhulst, 1996; Verboven & Visser, 1998). Such a decrease in general body condition as the season progresses may increase nestling susceptibility to parasitic infection, explaining the observed pattern. In addition, the optimum conditions for oocyst sporulation –which are essential for infectivity– are temperatures between 28 and 32°C and relative humidity between 35 and 60% (Merck *et al*., 2010; Malo, 2013), and poultry studies have shown that oocyst numbers in the environment rapidly decrease when the humidity is high (Clayton & Moore, 1997; Wilson *et al*., 2019). In our study site, maximum daily temperatures may increase from 21.7°C for first-cohort broods to 30.8°C in the second ones, and rainfall decreases significantly as the breeding season progresses, which may favour the persistence of oocysts in the environment.

The present work provides evidence of the relationship between changing environmental conditions throughout the breeding

season, and both the prevalence and intensity of coccidial infection in starling nestlings. However, future studies should examine the link between morphological and physiological variables that explain the variability in coccidial infection between individuals and clutches, as well as its potential impact on hosts at early life.

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