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Caution on the assessment of intestinal parasitic load in studying parasite-mediated sexual selection: The case of Blackbirds coccidiosis

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ABSTRACT

The parasite-mediated sexual selection (PMSS) theory has led to an increasing number of experimental studies essentially focussed on blood parasites. Currently, more research is being carried out on intestinal parasites in relationship to this theory. Before testing the theory with gastrointestinal parasites, it is important: (i) to determine an optimal research methodology to obtain an accurate assessment of parasite burden and (ii) to have information about life-history traits of the parasite to interpret data appropriately. In this study, we present data on oocyst output of *Isospora turdi* in the faeces of blackbirds (*Turdus merula*) that illustrate the importance of developing methods that are relevant for a particular model system, instead of relying on existing methods that may work in other systems. Our results show that: (i) a single droplet of faeces will accurately indicate the parasitic load in blackbirds, (ii) oocyst shedding varies greatly within and between days, (iii) the course of infection is characterised with two successive peaks of oocyst shedding, (iv) infection lasts approximately 1 month and (iv) there is no effect of sex, size of infective dose or re-infection on the course of infection. We discuss the practical implications of these results in determining the reliability of, and in avoiding erroneous conclusions about, PMSS when using intestinal parasites as models. In particular, we emphasise that numerous measurements must be performed on different days. In addition, faeces must be collected at a particular time of day because there is a strong temporal variation in oocyst shedding. Finally, a standardised methodology that has been developed for a particular host–parasite assemblage does not necessarily work with other biological models. Together, our results should encourage future investigators to identify an accurate methodology for assessing parasitic load as a first step before testing hypotheses associated with the PMSS theory.

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1. Introduction

The parasite-mediated sexual selection (PMSS) theory posits that females who choose healthy males as mates would have a selective advantage either through direct benefits (i.e. avoidance of parasites that could infect them and their offspring or healthier males which would provide better resources) or indirect benefits (i.e. production of offspring which would be more resistant to parasites by obtaining genes for parasite resistance) (Hamilton and Zuk, 1982; Møller et al., 1999). A key measurement in these studies is the infection status of the host (parasitised versus non-parasitised) or the parasitic load (number of parasites that a single host harbours) of males (e.g. Møller, 1990 and references therein; Willis and Poulin, 2000; Martin and Johnsen, 2007; Aguilar et al., 2008; Ezenwa and Jolles, 2008). This task appears very simple when researchers work with ectoparasites which can be easily counted (Borgia, 1986; Borgia and Collis, 1989). However, endoparasite load has proved more challenging to quantify. The most commonly used method is the examination of blood smears for blood parasites (Figuerola et al., 1999; Merilä et al., 1999) and/or the examination of faecal material for intestinal parasites (Costa and Macedo, 2005; Aguilar et al., 2008).

There are a number of factors that may influence the intensity of parasite load such as the sampling day (Giver et al., 2000; Villana et al., 2006), the time of sampling during the day (Brawner and Hill, 1999; Misof, 2004), phase of the infection (Giver et al., 2000; Otterstatter and Thomson, 2006), and the quantity of infective stages ingested by the host (Woolhouse et al., 1991; Williams, 2001). Consequently, and as pointed out by previous authors (Brawner and Hill, 1999; Misof, 2004; Villana et al., 2006), infections will not necessarily be detected with the same probability and the same intensity at all times. A few studies have dealt with the reliability of methods applied to estimate both parasite prevalence and intensity (Weatherhead and Bennett, 1991, 1992; Jovani and Tella, 2006; Marques and Cabral, 2007).

The great majority of studies on sexual selection and the effect of parasites on host life-history traits in birds have focused on

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blood parasites. Our knowledge of the interaction between blood parasites and their hosts and the reliability of techniques to estimate blood parasite intensity are now well developed (Valkiunias, 1997; Figuerola et al., 1999; Merilä et al., 1999). This knowledge is mainly due to intensive debate stimulated by concerns about the methodology used to assess parasite load (e.g. Cox, 1989). However, in contradiction to blood parasites, our knowledge of intestinal parasites remains fragmentary and more studies such as the one published by Hõrak et al. (2006) are needed to understand the relationship between intestinal parasites and their hosts. To date, our understanding of the evolutionary consequences of infection for hosts are limited by a paucity of information on the ecology of the parasites.

Blackbirds are naturally infected with intestinal parasites from the genus *Isospora* (Protozoa: Apicomplexa) (Misof, 2004). Two species have been reported: *Isospora lacazei* and *Isospora turdi* (Pellerdy, 1974). The asexual phase of these parasites occurs in the intestinal epithelium of the host, where one or more asexual multiplication cycles are completed before sexual development occurs (Olsen, 1974). Oocysts produced during the sexual phase are released into the host's intestinal tract and are passed in the faeces. Birds are infected orally when ingesting sporulated oocysts released in the faeces of an infected host. Due to the sexual phase, parasites originating from different hosts individuals are genetically diverse and thus could be considered as different strains (Hõrak et al., 2006).

Misof (2004) has shown that adult blackbirds shed coccidian oocysts predominantly in the afternoon, but did not provide more accurate information. In addition, Dolnik (2006) described a method for the quantification of coccidia oocyst output in a passerine bird: blackcaps (*Sylvia atricapilla*). Dolnik (2006) suggested that a single measurement of oocyst production at a specific time of day is sufficient to assess coccidian load, and that only one faecal droplet per bird is needed for analysis. Before applying these methods in blackbirds, it seemed important to check that these methodological tools developed for one particular assemblage of hosts and parasites also work in our system.

The first goal of the present study was to collect information in order to determine how to obtain an accurate assessment of coccidian load in blackbirds. To do this, we assessed variation in measurements depending on the time of day (more precisely than "afternoon", Misof, 2004) when the faeces were collected. We also quantified the number of days of collection required to obtain a reliable measurement of the parasitic load of infected blackbirds. In order to do this, we kept infected birds under controlled conditions and screened the dynamics of coccidia output during each day and between different days.

The second goal of this study was to better understand which parameters cause variation in *Isospora* infections in wild populations of blackbirds. To achieve a better understanding of the transmission dynamics, we wanted to determine: (i) the course of an *Isospora* infection over time in individually housed blackbirds under controlled conditions after an experimental infection, (ii) the effect of the number coccidian oocysts ingested by uninfected hosts on the establishment of the infection, (iii) whether males and females differed in their ability to resist and eliminate an infection. Comparative studies which have investigated sex-biased parasitism have reached the conclusion that males tend to have significantly higher parasite intensities than females (see Klein, 2004 for a review). We also wanted to determine (iv) if a first encounter with *Isospora* confers protective immunity against subsequent infection, given that re-infections are thought to occur frequently in the wild (Pedersen et al., 2003; Hõrak et al., 2006). It is particularly interesting to know whether protective immunity occurs in this species. To address this question, we experimentally infected uninfected blackbirds (male and female) or male birds that had received an infection 2 months previously with different doses (low, medium and high), of a natural strain of *Isospora* (i.e. parasites originating from only one host individual, see Hõrak et al., 2006 for a detailed study). We then followed the dynamics of the establishment of the infection by screening the number of oocysts shed in faeces every 2 days for 1 month.

2. Materials and methods

The work conforms to French legal requirements, and to accepted international ethical standards, including those relating to conservation and welfare, and to the journal's policy on these matters.

2.1. Trapping and general maintenance

Adult blackbirds (*Turdus merula*) (2 years old and older) were caught using mist-nets in the Botanical Garden of Dijon (France, 47°19′N, 5°02′E) from January to February, 2006. After capture, birds were housed in outdoor aviaries (220 × 150 × 250 cm) and fed ad libitum with food for large turdid species (COFNA) and tap water. Birds were maintained on a natural light cycle. One week before beginning experiments, all birds were placed in individual outdoor cages (69.5 × 44.5 × 82.5 cm) and continued to be fed as previously. Water and food were renewed each day. All the following experiments took place from February to the end of July, 2006.

2.2. Methods used to assess Isospora load in blackbirds

In our blackbirds, only one *Isospora* sp. was found and was identified as *I. turdi* according to the descriptions of Schwalbach (1959) and Svobodová (1994). To determine individual parasite loads, an aluminium sheet was placed on the bottom of the cage to collect faeces. All fresh faeces present on the aluminium sheet were placed in a Falcon tube for transportation to the laboratory. After homogenisation, we weighed 1 mg of faeces using an electronic balance which we then put into a 15 mL Falcon tube. Fourteen millilitres of the solution of Sheater (45% sugar) was added and we gently homogenised the solution. We sampled 600 μL of this solution, and used a McMaster chamber to count oocysts. The preparation was left for 10 min before counting, allowing the oocysts to float to the top. All the oocysts under the grid of each chamber in the McMaster were counted using 10× magnification. The average of two counts from each individual faecal sample was used as the estimate of daily oocyst production rate and their concentration was expressed as number of oocysts per gram of faecal sample.

2.3. Evaluation of a standardised method

Our first experiment was to designed to test whether the methods described above produce repeatable results. We performed two tests on 29 infected birds. First, we counted the number of oocysts contained in the same sample of 1 g of faeces twice. To do this, 1 g of faeces was placed in a Falcon tube with 14 mL of Sheater solution. We took two samples of 600 μL from the same tube after homogenisation and we counted the number of oocysts. Second, we tested whether the number of oocysts was the same between two different samples of 1 g of faeces shed by the same individual. After homogenisation of the faeces, we prepared two Falcon tubes with 1 g of faeces in each, from the same individual, and we counted the number of oocysts using the same technique (see Section 2.2).

The goal of our second experiment was to determine whether the *Isospora* load was the same in each droplet shed by one specific individual during a given period of time. From 15 infected males, we collected five droplets shed by the same individual between 17:00 h and 18:00 h (see Section 3 for a justification of this period

in the day). We put the droplets in separate Falcon tubes and we measured the *Isospora* load in each droplet using the same procedure as described previously. However, in this experiment, most droplets were less than 1 mg. Therefore, we adjusted the volume of Sheater solution added to the droplet in order to keep concentration constant (i.e. 1 mg of faeces for 14 mL of Sheater solution).

2.4. Diurnal periodicity and day-to-day variation in excretion of Isospora oocysts by blackbirds

To determine the precise time of day at which the faeces could be collected to determine the *Isospora* load (i.e. to determine the peak of appearance in *Isospora* oocysts), faeces of 10 wild-caught infected birds from a natural population were collected at several different hours of the day. In the evening, cages were carefully cleaned. The first collection occurred at sunrise (i.e. faeces shed during the night). Afterwards, faeces were collected every 2 h until sunset (approximately 21:00 h in July). Consequently, eight samples were collected for each individual. For each sample, we measured the *Isospora* load using the same procedure as described above (see Section 2.2).

To assess the extent of day-to-day variation in *Isospora* load, we kept the 10 infected birds in individual outdoor cages and we collected faeces every 2 days for 18 days when the peak of oocyst excretion occurred (between 16:30 h and 19:00 h, see Section 3). We determined the *Isospora* load using the method described previously (see Section 2.2).

2.5. Dynamics and variation of experimental infection

In order to track the dynamics of infection, we experimentally infected blackbirds with a strain of *Isospora* (i.e. parasites originating from only one host individual). We collected faeces of blackbirds during the peak period of oocyst excretion (between 16:30 h and 19:00 h, see Section 3) in order to quantify the numbers of *Isospora* at three different time points: (1) the day of the experimental infection to assess the baseline intensity of infection (day 0), (2) the following day to assess if a passive release of sporulated oocysts had occurred (day 1) and (3) every 2 days for 1 month to track the course of the infection (day 3–29). To obtain uninfected blackbirds, we used the same procedure as described in Baeta et al. (2008).

To obtain *Isosporan* oocysts for the experimental infection, we collected faeces from one naturally, heavily infected adult male blackbird. This bird was caught 2 weeks before the beginning of the experiment and housed alone in an outdoor cage (69.5 × 44.5 × 82.5 cm). An aluminium sheet was placed on the bottom of the cage to collect infected faeces. The faeces were collected each day for 3 days and placed in a solution of potassium dichromate diluted in water (2.2%). To obtain oocyst sporulation, a thin layer of this suspension was exposed to air for at least 3 days. Oocysts were then washed with distilled water to remove the potassium dichromate from the suspension. Four washing procedures were performed sequentially following a standard protocol. One millilitre of the suspension of potassium dichromate was mixed with 9 mL of distilled water and centrifuged at 700*g* for 10 min. The supernatant was removed after centrifugation and the residue with oocysts was mixed again with 9 mL of water. After the last washing procedure, the oocysts were floated by dissolving 1 mL of residue in 10 mL of Sheater solution. A McMaster chamber was used to count viable, fully sporulated oocysts. Six hundred microlitres of this solution were deposited in the chamber and the fully sporulated oocysts were counted. Their concentration was expressed as number of oocysts per gram of faeces. Two counts were conducted, and the mean number of sporulated oocysts was used as the estimate of oocysts abundance.

To assess the potential effect of dose on the establishment of the infection, male blackbirds were randomly assigned to receive different doses: 9000 (low dose), 14,000 (medium dose) or 18,000 (strong dose) sporulated oocysts. This dose for infection was calibrated according to the dose used by Hõrak et al. (2004) in greenfinches (*Carduelis chloris*) and should be biologically meaningful in blackbirds, given our recent observations that 0.1 g of blackbird faeces can contain more than 10,000 oocysts (Moreau, unpublished data). Each individual from the infected groups was inoculated orally with isosporan oocysts diluted in 1.5 mL of water at day 0. Birds were inoculated with a cannula that was gently introduced into the throat to reach the oesophagus. We used 14 male blackbirds in each group. Birds from the control group were inoculated with an equal volume of water ($n = 11$). To evaluate the effect of sex in the establishment of infection, we also infected 10 females with the medium dose. To evaluate the effect of first or second infection, we infected 10 males that had already received an experimental infection of another strain (i.e. parasites originating from another host individual) of *Isospora* 2 months previously. Given that we had access to the experimentally infected birds in individual outdoor cages, we also determined whether these different experimental treatments could have an effect on the diurnal periodicity of oocyst shedding. For that, we used the same procedure previously described (see Section 2.2) once individuals became infected (approximately 15 days p.i., see Section 3).

2.6. Statistical analysis

The statistical tests were performed using the JMP software package (Version 3.2.2, SAS institute Inc.). Two-tailed tests of significance were used throughout. The distributions of the parasitic counts were often skewed and the data were log transformed to achieve approximate normality (Shapiro-Wilk's test). For evaluation of the counting method and the analysis of day-to-day variation, we calculated repeatability (τ) as the intra-class correlation coefficient using variance components derived from one-way ANOVA and following Lessells and Boag (1987). We also checked the homogeneity of variances using Levene's test. For the analysis of both diurnal periodicity and dynamics of experimental infection, we used a repeated-measure ANOVA to check the effect of sampling time on number of oocysts shed. To analyse if sex (male versus female), dose (low versus medium versus strong) and re-infection (first versus second infection) had an effect on the dynamics of the infection, we treated these factors as covariables in the repeated-measure ANOVA.

3. Results

3.1. Evaluation of the standardised method

There were no statistically significant differences in the number of oocysts counted between two sub-samples of 600 μL for the same faecal sample (intra-class correlation coefficient on log transformed data, $n = 29$, $\tau = 0.99$, $P < 0.0001$) or between two samples of 1 g of faeces (intra-class correlation coefficient on log transformed data, $n = 29$, $\tau = 0.99$, $P < 0.0001$). The variation in number of oocysts counted in different droplets within 1 h was greater between than within individuals (intra-class correlation coefficient on log transformed data, $n = 15$, $\tau = 0.75$, $P < 0.0001$).

3.2. Diurnal periodicity in Isospora oocyst output

A repeated measure ANOVA showed that the number of oocysts in the faeces was dependent on the time of the day but was not different among experimental groups (ANOVA with repeated measures: effect of group: $F_{5,54} = 0.99$, $P = 0.43$, effect of time:

$F_{7,48} = 14.01$, $P < 0.0001$, effect of their interaction: $F_{35,260} = 1.25$, $P = 0.17$) (Fig. 1). Before 12:00 h, none of the blackbird faeces contained isosporan oocysts. After 12:00 h, oocyst count for blackbirds increased at each sampling time to reach a peak between 17:00 h and 19:00 h. After 19:00 h, oocyst counts started to decrease. During the night, there were some oocysts shed (Fig. 1).

3.3. Day-to-day variation in excretion of *Isospora* oocysts in blackbirds

Intra-individual variation in the quantity of oocysts shed by isolated blackbirds over 10 days' examinations was high (Fig. 2). The variation in number of oocysts shed was greater within individuals than between individuals with a very low correlation coefficient (intra-class correlation coefficient on log transformed data, $n = 10$, $\tau = 0.08$, $P = 0.10$).

3.4. Dynamics of experimental infection and factors of variation

Before the start of infection, the level of infection was very low and did not differ between groups (Anova: $F_{5,72} = 0.72$, $P = 0.61$). Control birds did not show any variation in the number of oocysts shed and had different dynamics of oocysts shedding from other groups (ANOVA with repeated measures: effect group: $F_{5,67} = 3.43$, $P = 0.008$, effect time: $F_{14,54} = 7.11$, $P < 0.0001$, effect of their interaction: $F_{70,290} = 1.38$, $P = 0.03$) (Fig. 3). If we excluded the control group from the analysis, the number of oocysts shed in the faeces was strongly dependent on the time of the day but was not different among various experimental groups (ANOVA with repeated measures: effect of group: $F_{4,57} = 0.48$, $P = 0.75$, effect of time: $F_{14,44} = 6.94$, $P < 0.0001$, effect of interaction: $F_{56,188} = 1.07$, $P = 0.36$) (Fig. 3). Therefore, there was no difference in the dynamics of infection: (i) between males and females, (ii) between males which had a previous infection and naive males, and (iii) between males that received different infection doses. In each group, oocysts were first observed on day 3 p.i. Following the first appearance, the number of oocysts increased dramatically and then dropped to return to the initial level at day 7 p.i. Oocyst counts then increased rapidly again until a peak level was reached from 9 to 13 days p.i. Subsequently, the number of oocyst declined slowly in all infected blackbirds and returned to the previous levels of infection within 3 weeks. Marked individual variations were observed between blackbirds around this general trend.

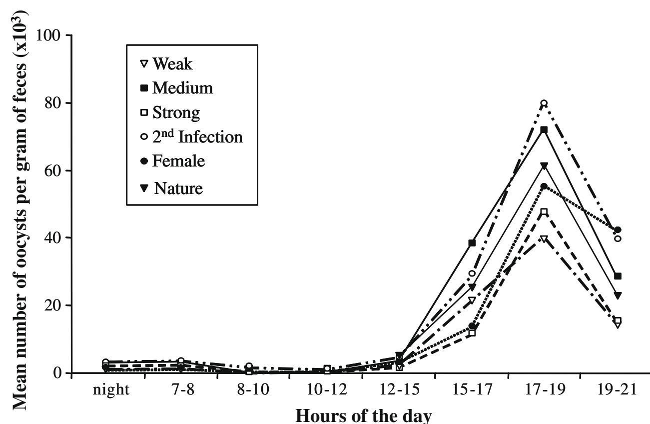

Fig. 1. Mean number of oocysts per gram of faeces collected at different times of day for each group. For clarity, error bars are omitted.

4. Discussion

Our results indicate that counting *Isospora* oocysts with a McMaster chamber produces highly repeatable results, and we conclude that this method can be used with confidence. Further, we found that a single droplet can provide an accurate measure of the parasitic load in blackbirds. This was similar to the results reported by Dolnik (2006) in blackcaps (*Sylvia atricapilla*). This result should be encouraging for researchers working on natural populations of blackbirds. In the field, it is easier to follow individuals and collect a small droplet when they defecate (e.g. Misof, 2004) than to collect a large amount of faeces which requires that individual birds be captured. Unfortunately, even if a single droplet reflects the precise *Isospora* load of the individual, this information is only informative for the days when the samples are collected. Our results clearly show that the production of oocysts in naturally infected *T. merula* varies strongly between successive days over a period of 10 days. This finding conflicts with the results of Dolnik's study of blackcaps (2006), where the author found that the variation in intensity of isosporan infection was relatively stable over time and concluded that a single measurement of oocyst production could be used to characterise individual coccidian infection status. Taken together, these studies strongly emphasise that conclusions drawn for one species are not necessarily applicable to others. This significant day-to-day variation of egg output has been carefully documented in several other parasitic infections (Engels

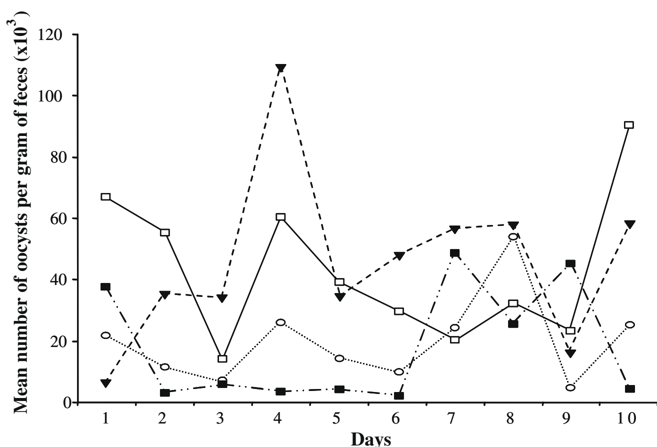

Fig. 2. Variation in the production of oocysts in *Turdus merula* over a period of 10 consecutive days of sampling. For the sake of clarity, only four individuals are shown.

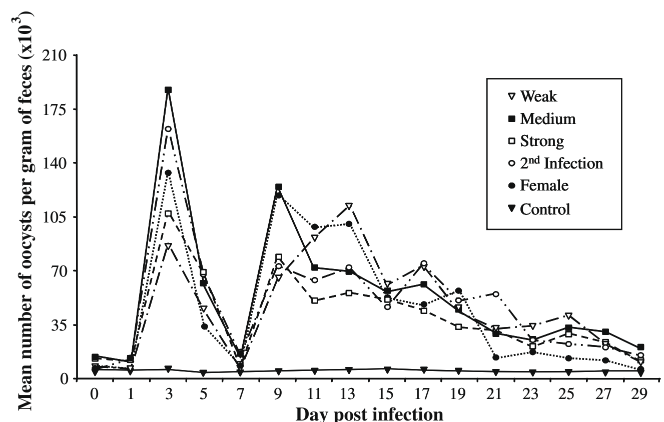

Fig. 3. Mean number of oocysts per gram of faeces collected on different days p.i. for each group. The experimental infection occurred at day 0. For clarity, error bars are omitted.

et al., 1996; Giver et al., 2000; Otterstatter and Thomson, 2006; Villana et al., 2006) and may be very common in wild populations, given that when we sampled naturally infected individuals, each bird was in a different phase of infection. Furthermore, several strains of coccidia can occur in host faeces simultaneously. As it is impossible to distinguish those, the oocysts found in faeces likely belong to several different infections, each one at a different stage of infection. In the future, it would be interesting to establish how many days of collection are necessary to obtain an accurate assessment of infection. Simulation procedures would certainly very useful to determine a threshold for a reliable judgement of the parasitic load (Marques and Cabral, 2007).

In addition to the day-to-day variation in oocyst count, our results indicate that the time of the day when faeces are collected has a very strong effect on the number of *Isospora* oocysts found in faeces. Blackbird faeces contain almost no oocysts in the morning. Shedding increases from the middle of the day onward and reaches a peak in the late afternoon. This circadian shedding has previously been reported in several species of birds, in which peak shedding is in the late afternoon (Boughton, 1933, 1988; Kruszewicz, 1995; Brawner and Hill, 1999; Hudman et al., 2000; Brown et al., 2001; López et al., 2007). The proximal factors that trigger oocyst discharge are not yet known but melatonin could play an important role in the regulation of parasite release rhythms. Martinaud et al. (2008) have experimentally demonstrated that the peak in late afternoon is an adaptation to prevent the desiccation of oocysts in order to increase the probability of survival in the external environment.

Whatever the mechanisms behind this circadian shedding, our results are consistent with those described by Misof (2004) but emphasise the need for greater precision when recording the time of day at which samples are taken. Clearly, there is a large difference in the number of *Isospora* oocysts shed between the beginning of the afternoon (12:00–16:00 h) and the end of the afternoon (16:00–19:00 h). If the goal is to make meaningful comparisons of *Isospora* loads between individuals, sampling must be done at the same time of day for all birds. Only a few hours difference between individuals could have a significant effect on the reliability and validity of data.

The significant day-to-day variation in oocyst shedding can be explained in part by the phase of the infection (Giver et al., 2000; Otterstatter and Thomson, 2006). We found a clear pattern in the course of infection in all experimental groups. In all cases, the number of oocysts shed increased dramatically 3 days after the experimental infection, which represents a very short prepatent period. All *Isospora* prepatent periods reported in the literature are longer than this: around 8 days for *Isospora* infecting the Snow Bunting (*Plectrophenax nivalis*) (Dolnik and Loonen, 2007), from 4 to 9 days in canaries (*Serinus canarius*) (Box, 1977), from 6 to 7 days in greenfinches (*Carduelis chloris*) (Mehlhorn et al., 1986; Hõrak et al., 2007). After this prepatent period, the levels of oocysts shed decreased to the initial level before reaching a new peak around 10 days p.i. Oocysts continued to be passed in the faeces for 3 weeks after the appearance of the second peak corresponding to the patent period which is also extremely variable according to the species (e.g. Box, 1977). Overall, the infection lasted approximately 1 month. We cannot yet explain why both prepatent and patent period are highly variable between species, but the life cycle of *Isospora* and their location in the host's intestinal epithelium has a strong effect on the duration of each stage. Whatever the underlying mechanisms, our results and those of colleagues demonstrate that each *Isospora* species has a specific life cycle of different duration. *Isospora* spp. are known to have a high degree of specificity and some authors argued that each host species harbours one *Isospora* species (Cerna, 1973; Box, 1980; Dolnik, 2002). This means that the results found in one host species are not directly transferable to another. Therefore, preliminary studies of a specific host–parasite life cycle are a necessary step in designing experiments and correctly interpreting results. For example, if we want to know whether an experimental infection affects host body condition or the immune response induced by the parasite, it seems necessary to standardise the measure according to the course of infection (e.g. Hõrak et al., 2004). The expected results would not be the same if the condition indices were measured at the beginning, middle or the end of the infection.

We were unable to detect any differences in the course of *Isospora* infection (in terms of the number of parasites shed and the number of days for which infection occurs) that were attributable to sex, to infection dose and to re-infection. From our results, we can conclude that immunological responses against infection are similar between male and females. These results may be surprising given that testosterone is known to have immunosuppressant effects, while oestrogen may stimulate the immune system (see Klein, 2004). However, our results should be interpreted cautiously since we infected individual birds only once during one particular part of their life cycle. It may be that the results would be different during another period. Therefore, both sexes contribute to the propagation of the infection in the host population. In addition, we did not find that the number of oocysts shed was dependent on the infective oocyst dose. It seems that the crowding effect described in *Eimeria* (Williams, 2001) is not applicable in this system, at least in the range of doses tested. We speculate that the number of infective oocysts that successfully establish in epithelial cells and undergo a multiplication cycle is the same whatever the number of sporulated oocysts ingested. In this context, the number of the available intestinal host cells may act as a threshold as has been suggested previously (Johnston et al., 2001). Additionally, we found that previous encounters with an *Isospora* strain do not confer protective immunity against subsequent infections with other strains. This result is consistent with studies in greenfinches (Hõrak et al., 2006) and more generally with species of *Isospora* (Long, 1982).

This study emphasises several factors that should be taken into account in order to obtain reliable data on parasitism to properly test hypotheses concerning the PMSS theory.

Firstly, it appears absolutely necessary to carry out preliminary studies to determine how to correctly assess parasite load for a given species. In particular, the variation between days in *Isospora* oocyst counts represents a challenge for their use in comparing counts between individuals or populations. Our results show that single measurements do not provide robust measurements of parasite load. We recommend that repeated measurements on different days should be performed to obtain reliable measures of a bird's *Isospora* load and to account for possible daily variation (see Dawson and Bortolotti, 1999; Hõrak et al., 2004 for examples). This procedure requires that birds be repeatedly caught or kept in captivity.

Second, the large within-day variation of oocyst count we found here suggests that researchers interested in comparing *Isospora* load between individuals should pay attention to this circadian rhythm. Samples should always be taken at the same time of day. If sampling is not standardised it becomes impossible to compare *Isospora* load between different individuals. In blackbirds, we recommend assessment of *Isospora* load between 17:00 h and 19:00 h when the peak output occurs.

Finally, even if data are available for other biological models, our study showed that a standardised method developed on one particular host–parasite assemblage does not necessarily work on another model (Dolnik, 2006). As the circadian rhythm is probably the result of long-term co-evolution between parasite and host, and as the selective pressures are not the same between species, we cannot be certain that the time of sampling for one species will

be accurate for another species. Therefore, a first step must be to determine when the parasitic load can be assessed accurately.

Based on the results of this study, we believe that it is crucial for researchers studying PMSS theory to consider the importance of data collection methods. Studies should begin by gathering as much information as possible about the life cycle of the parasite, and then determining a sampling strategy that is relevant for the particular host–parasite system.

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