Circadian variation in shedding of the oocysts of *Isospora turdi* (Apicomplexa) in blackbirds (*Turdus merula*): An adaptive trait against desiccation and ultraviolet radiation

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**A B S T R A C T**

Many parasite species spend part of their life cycle in the external environment waiting for a new host. Emergence of parasites often occurs once a day, which may help to minimise mortality in an inhospitable environment and increase transition rates. Many intestinal parasites in birds are released in faeces only in the late afternoon. However, the adaptive significance of this pattern is unclear. One hypothesis is that a particular time of emergence may prevent parasite desiccation and therefore increase the parasite’s life expectancy in the external environment. We tested this hypothesis experimentally using the blackbird (*Turdus merula*) infected with *Isospora turdi* (Protozoa: Apicomplexa). We found that short exposure of faeces to natural sunlight has a dramatic effect on oocyst survival. This appears to be due to the effect of warmth and ultraviolet (UV) radiation with UVB waves being more damaging than UVA. Oocysts contained in faeces shed in water are protected from the effect of sunlight. Together, these results suggest that the release of oocysts in the late afternoon is an adaptive trait to avoid desiccation and UV radiation, thus reducing mortality of the oocysts in the external environment.

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1. Introduction

Parasites may spend a part of their life cycle outside the host when reproductive propagules are released from the host into the external environment, where they wait to be transmitted to a new host. Hence, the fitness of the parasite depends, at least in part, on its ability to resist adverse external conditions before transmission. From an evolutionary perspective, it is generally assumed that any trait increasing the transmission success of the parasite from one host to another would be selected for (see Bush et al., 2001 or Combes, 2001). One of the responses acquired by parasites during the course of evolution consists of synchronising their emergence from one host when encounters with the next host are most probable. One of the best-known examples is the cercarial-shedding pattern of various species of *Schistosoma* that coincides with the times when the definitive host is most likely to visit the aquatic habitat in which transmission will occur (Théron, 1984; Combes et al., 1994; Combes, 2001).

This phenomenon of diurnal periodicity is also observed in other parasite species, especially intestinal parasites from the genus *Isospora* (Protozoa: Apicomplexa) in birds, which release their oocysts in the late afternoon (Boughton, 1933; Grulet et al., 1982; Kruiszewicz, 1995; Brawner and Hill, 1999; Dolnik, 1999a, 1999b; Hudman et al., 2000; Brown et al., 2001; Misof, 2004). These protozoans exhibit both sexual and asexual phases. In most species the asexual phase 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 passed in the faeces. To complete their development, oocysts have to sporulate (the infective stage) outside the host, and await a new host. Only oocysts that have sporulated are able to infect a new host.

While the phenomenon of diurnal periodicity in oocyst release of *Isospora* in birds is well known, the adaptive significance has not been tested experimentally. However, two hypotheses have been proposed to explain this circadian variation in oocyst output in *Isospora* (e.g. Dolnik, 1999a, 1999b; McQuistion, 2000; Misof, 2004). Firstly, oocyst release corresponds to a peak of feeding activity in birds. As many individuals share the same feeding patch, we may then suppose that oocysts released in the feeding area will have a higher probability of transmission. However, birds are known to have two feeding peaks, one in the morning and one in the evening, so it is difficult to understand why oocysts do not also appear in the morning. Second, oocysts are generally thought to be relatively resistant to environmental factors (such as temperature, relative humidity, etc). Nevertheless, it has been reported that desiccation can reduce the infectivity of the oocysts of *Eimeria* in poultry (Duncan, 1959; Marquardt et al., 1960; Schneider et al., 1972).
Therefore, the appearance of *Isospora* oocysts late in the afternoon could be an adaptation to prevent their desiccation under natural conditions and ensuring that oocysts can reach the infective stage in a very short time.

In this study, we aimed to explain the temporal pattern of oocyst release by testing oocyst resistance to desiccation when exposed to daylight conditions. As a model system we used the common blackbird (*Turdus merula*), because it harbours *Isospora turdi* (Schwalbach, 1959; Svobodová, 1994) and they are very abundant in urban parks (Misoř, 2004; Baeta et al., 2008). First, we assessed the dynamics of appearance of infective oocysts for 24 h after the faeces had been shed by their hosts in the late afternoon. Second, to test oocyst resistance to daylight conditions, we placed fresh faeces containing oocysts either under natural sunlight or in shadow and we assessed oocyst survival. Third, we further examined the potential factors that contribute to oocyst death by experimentally testing the effect of heat and ultraviolet (UV) radiation (UVA and UVB). Finally, as blackbird faeces are frequently found in water, we examined whether water has a protective effect on oocyst survival.

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 male blackbirds (2 years old and older) were caught using mist-nets in urban parks of Dijon (France, 47°19′N, 5°02′E) during spring, 2007. After capture, birds were kept in outdoor aviaries (220 × 150 × 250 cm) and fed ad libitum with food for large turdoid species (COFNA) and tap water during the period of captivity. Water and food were renewed each day. A preliminary screening of the faeces of the birds allowed us to detect infected individuals. All infected birds were then placed in individual outdoor cages (69.5 × 44.5 × 82.5 cm) and fed as described above and were used for the subsequent experiments that took place in June 2007.

2.2. Experiment 1: dynamics of appearance of infective (=sporulated) oocysts after the faeces have been shed

We first determined when the first sporulated oocysts appeared under natural conditions. The faeces of 10 infected blackbirds were collected from 16:30 to 19:00 h by placing an aluminium sheet on the bottom of the cage. Faeces were sampled every hour. The faeces of 10 infected blackbirds (the same individuals used in experiment 1) were collected from 16:30 to 19:00 h by placing an aluminium sheet on the bottom of the cage. Faeces were sampled every hour. Therefore, the appearance of *Isospora* oocysts late in the afternoon could be an adaptation to prevent their desiccation under natural conditions and ensuring that oocysts can reach the infective stage in a very short time.

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2.3. Assessment of oocyst viability

Viability of oocysts was assessed by counting the proportion of sporulated oocysts after a short period in a solution of potassium dichromate (Duncan, 1959). The sampled faeces were put in a small vial with 5 mL potassium dichromate solution diluted in water (K₂Cr₂O₇, 2%) and broken into small pieces with a wooden dowel. A solution of 2% K₂Cr₂O₇ was found to give consistently high sporulation at a room temperature range (20–26 °C) (Duncan, 1959; Moreau, personal observation). The vials were left open for 48 h to allow oocysts to sporulate, which is adequate time for oocysts infecting blackbirds to sporulate (Moreau, personal observation). Non-sporulated oocysts were considered to be dead. After 2 days, the oocysts were then washed with distilled water to remove the potassium dichromate from the suspension. Four washing procedures were performed sequentially. One millilitre of the suspension of potassium dichromate was mixed with 9 mL of distilled water and centrifuged at 700g for 10 min. The supernant 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 in order to assess the proportion of sporulated oocysts in each glass vial, following the same procedure as described above.

2.4. Experiment 2: natural sunlight effect on oocyst viability

The faeces of 10 infected blackbirds (the same individuals used in experiment 1) were collected using the same procedures. Homogenised faeces were placed on a plate in the refrigerator overnight. Keeping blackbird faeces in a refrigerator has no effect on the viability of oocysts (Moreau, personal observation). The following morning at 10:00 h, we took 0.5 mg from each faecal sample and we exposed the plate with the rest of faeces to moderate natural sunlight (partly cloudy). Afterwards, we collected 0.5 mg of each faecal sample 1, 2, 3 and 4 h later. The viability of the oocysts was then determined using the procedure described above.

2.5. Experiment 3: effects of UV light, artificial light and temperature on oocyst viability

In order to find the potential factors that contribute to oocyst death, we performed different experiments following the same general procedure. We collected and homogenised the faeces of 15 infected blackbirds. For this experiment, we used different individuals than those used in the two previous experiments. Faeces from these 15 blackbirds were then split in two equal portions in two small vials and kept in the refrigerator overnight. The next morning, the first half of the faeces was exposed to one treatment (see below) and the second half was used as a control. In this way, we had 15 replicates for each experiment. We repeated the collection of faeces from these 15 infected blackbirds every day in order to perform all the following experiments. The sequence of experiments was randomized.

Our primary goal was to measure the effect of UV radiation and temperature on the viability of oocysts. In a first experiment, we exposed the faeces of infected blackbirds to either UVA or UVB radiation for either 4 or 8 h. UVA and UVB exposures were conducted using fluorescent lamps (Fisher Scientific Bioblock, Strasbourg, France) and faeces were placed 30 cm below the lamps. The lamps emitted wavelengths at 312 nm (UVB) and 365 nm (UVA). To prevent overheating, this experiment took place in room with a temperature of 14 ± 1 °C. In a second experiment, we exposed the faeces to shade under natural conditions for either 4 or 8 h. In a third experiment, we exposed the faeces 30 cm below an artificial...
light (150 lux) for either 4 or 8 h. In a fourth experiment, we placed faeces in a drying oven at 37 °C for either 6, 8 or 10 h. Finally, to determine the potential effect of water on oocyst resistance against UV radiation, we placed faeces in 20 mL of water and we exposed the vial to natural sunlight for either 4 h or 8 h. The vial was open to allow direct exposure of the contents to sunlight. Control faeces were stored for the same time periods as treated faeces in room with a temperature of 20 ± 1 °C, except for UV exposure where control faeces were stored in the same room as treated faeces (14 ± 1 °C). Immediately after the experiment, we placed control and treated faeces in 5 mL of K2Cr2O7 to assess the proportion of sporulated oocysts (see section above) and to compare the proportion of sporulated oocysts between control and treated faeces. We present the results as the change in the percentage of sporulated oocysts: \((N - N_0)/N_0 \times 100\), where \(N = \%\) oocysts sporulated in treated faeces, \(N_0 = \%\) oocysts sporulated in control faeces. Consequently, negative values indicate a decrease in the percentage of sporulated oocysts in treated faeces.

2.6. Statistical analysis

Statistical tests were performed using JMP software (Version 3.2.2, SAS institute Inc.). Two-tailed tests of significance were used throughout. The assumptions for parametric analyses were met for all tests (Shapiro-Wilk’s test for normality and Levene’s test for homogeneity of variances). For experiments 1 and 2, we used a repeated-measure ANOVA to check the effect of sampling time on the proportion of sporulated oocysts. For experiment 3, a decrease in the percentage of sporulated oocysts (relative to controls) was tested for statistical significance using a paired t-test. To check the effect of treatment duration, we used t-tests (4 versus 8 h) or ANOVA (6, 8 versus 10 h) followed by probable least-squares difference (PLSD) tests to see the difference between groups.

3. Results

The proportion of sporulated oocysts increased regularly with time to a maximum of 70% of sporulated oocysts in faeces at 18 h (repeated-measure ANOVA: effect of time \(F_{1,9} = 250.07, P < 0.0001\), Fig. 1). By 10 h, more than 20% of oocysts were already in the infective stage. The exposure to sunshine had a dramatic effect on oocyst viability (repeated-measure ANOVA: effect of time \(F_{1,9} = 874.82, P < 0.0001\), Fig. 2). Only 1 h of exposure reduced oocyst viability. After 4 h of exposure, only a few oocysts remained viable.

Exposure of the faeces in shade for 4 or 8 h had no effect on the viability of oocysts (shade 4 h: \(t_{14} = 1.31, P = 0.21\); shade 8 h: \(t_{14} = 2.00, P = 0.07\); no difference between 4 and 8 h: \(t_{28} = 0.92, P = 0.37\), Fig. 3a, black columns). Similar results were found for artificial light (artificial light 4 h: \(t_{14} = 1.86, P = 0.08\); artificial light 8 h: \(t_{14} = 0.68, P = 0.50\); no difference between 4 and 8 h: \(t_{28} = -0.76, P = 0.45\), Fig. 3a, white columns). Exposure of the faeces to UVA or UVB radiation reduced the proportion of viable oocysts (UVA 4 h: \(t_{14} = 5.60, P < 0.0001\); UVA 8 h: \(t_{14} = 5.66, P < 0.0001\); UVB 4 h: \(t_{14} = 6.92, P < 0.0001\); UVB 8 h: \(t_{14} = 9.41, P < 0.0001\), Fig. 3b). Eight hours of UVB radiation further decreased oocyst viability compared to 4 h, but the duration of UVA exposure had no effect (UVA: \(t_{28} = 0.59, P = 0.59\); UVB: \(t_{28} = 2.32, P = 0.03\), Fig. 3b). A longer exposure to high temperature (37 °C) reduced the viability of oocysts (6 h: \(t_9 = 4.52, P = 0.001\); 8 h: \(t_9 = 4.87, P < 0.0001\); 10 h: \(t_9 = 8.68, P < 0.0001\); difference between 4, 8 and 10 h: \(F_{2,29} = 11.37, P = 0.0003\), Fig. 4).

Blackbird faeces exposed to sunlight in water for 4 or 8 h did not show a significant difference in the viability of oocysts compared with controls (4 h: \(t_{14} = 1.27, P = 0.23\); 8 h: \(t_{14} = -1.17, P = 0.26\); no difference between 4 and 8 h: \(t\)-test: \(t_{28} = -1.71, P = 0.10\), Fig. 3c).

4. Discussion

The results of this study clearly show that (i) approximately 70% of oocysts contained in a blackbird’s faeces reach the infective stage within 24 h, (ii) sunlight has a dramatic negative effect on oocyst survival, (iii) which is mainly due to the effect of high temperature and UV radiation and (iv) faeces with oocysts shed in water are protected from the adverse effect of sunlight.

It is interesting to note that approximately 30% of the oocysts shed in blackbird faeces did not reach the infective stage after 24 h. This proportion is similar to the proportion found under ideal conditions of sporulation even after 72 h (Duncan, 1959; Moreau, personal observation). Therefore, approximately 30% of oocysts shed will never be infective. Interestingly, in faeces shed in the late afternoon, we found sporulated oocysts the following morning. Therefore, blackbirds foraging during the morning can ingest infective oocysts. This time-frame to reach sporulation seems unusual since Pellerdy (1974) mentioned that the process of sporulation takes between 48 h and 7 days depending on the species of Isospora.
Our results clearly show that brief exposure to sunlight can have a dramatic effect on oocyst viability. A single hour of exposure reduced the viability of oocysts by approximately 50%, and this further decreased with continued exposure. During our experiments, the sky was partly cloudy and cloud often masked the sun during the second half of the experiment. The decrease in oocyst survival may be more drastic with a cloudless sky. The proximal factors responsible for this reduction seem to be a prolonged exposure to UV light and the effect of overheating. Desiccation has already been reported to reduce the infectivity of the oocysts of *Eimeria* species (Duncan, 1959; Marquardt et al., 1960; Schneider et al., 1972). To our knowledge, this is the first time that this has been shown in an *Isospora* species. The role of UVA and UVB radiation is also very clear. Brief exposure to UV light strongly reduced the viability of oocysts. According to our experiments, the dose received by oocysts was similar to sunlight for 2 h and is consequently relevant to the conditions that oocysts may face. The negative effect of UV radiation has already been demonstrated on survival and infectivity of miracidia, sporocysts and cercariae of *Schistosoma mansoni* (Ariyo and Oyerinde, 1990; Ruelas et al., 2007). Our results indicate that UV light can have a significant effect on the transmission success of the parasite. Based on our knowledge of the effects of UV light on organisms, we may hypothesise that the absence of oocyst sporulation under UV light is due, at least in part, to damage to the structure and function of DNA (Friedberg et al., 1995). Indeed, the oocysts observed in the faeces irradiated with UVB or UVA wavelength underwent abnormal development. In addition, UVB radiation seems to cause more damage to *Isospora* oocysts than UVA. This result is consistent with the fact that UVB is at a higher energy level than UVA (Paul and Gwynn-Jones, 2003).

One of the most interesting results of our study was the fact that oocysts contained in faeces shed in water remained viable even during a long exposure to sunlight. This result means that the water protects oocysts against desiccation and UV radiation. It is likely that water reduced the amount of UV radiation reaching oocysts. The attenuation of solar UVB radiation ranges from several centimetres to >10 m and depends mainly on the percentage of dissolved organic carbon (Morris et al., 1995).

Together, these results clearly suggest that the peak of oocyst release late in the afternoon is new evidence for its adaptive significance to increase the probability of encounters between host and parasite by synchronising parasite release with the presence of the host. This “chronobiological” adaptation is especially well known in (i) trematodes for which the cercarial-shedding pattern coincides with the time schedule when the definitive host is present in the environment (Théron, 1984; Combes et al., 1994; Combes, 2001) and (ii) for some blood parasite species (such as *Wuchereria bancrofti* microfilariae) which appear in peripheral blood at specific times coinciding with vector feeding period (Hawking, 1967; Sasa, 1976; Combes, 2001). In the case of *I. turdi*, the peak of oocyst release late in the afternoon is an adaptive trait providing resistance to desiccation and UV radiation. If oocysts were released throughout the day, most would die very quickly due to exposure to sunlight. The release of oocysts in the evening ensures that most of those will sporulate the next morning and thus will be ingested by other blackbirds during the morning peak of feeding activity. If oocysts appeared in the morning, they would have to cope with sunlight during the whole day, and would
have an increased probability of dying. Therefore, the release of oocysts late in the afternoon enhances both survival and transmission of the parasite.

Isosporan coccidia are found in a wide variety of avian species and the temporal variation in shedding of coccidial oocysts is known in many birds species such as house sparrows (Boughton, 1933, 1988; Gruetl et al., 1982; Kruszewicz, 1995), house finches (Brawner and Hill, 1999), dark-eye juncos (Hudman et al., 2000), European Serin and Garden Warblers (López et al., 2007), greenfinches (Brown et al., 2001) and blackbirds (Misof, 2004). We believe this study is the first to describe an adaptive function of the temporal variation in oocyst shedding. Future work will investigate whether this adaptation against desiccation and UV radiation exists in other species. It will be particularly interesting to determine whether this circadian rhythm could result from co-evolution of parasites and hosts. For example, it would be feasible to test whether this parasite’s circadian rhythm varies between different geographic areas within the same host species. If this circadian rhythm is the product of natural selection that increases oocyst survival, we may hypothesise that oocysts will appear in faeces at different times depending on the latitude of the geographical area. For example, we may expect that blackbirds living further south (with more intense sunlight) will have a peak later in the afternoon allowing a greater resistance to intense sunlight. Other questions raised by our results are: whether blackbirds that inhabit open areas are more likely to shed oocysts in strongly circadian patterns than those that live in shaded habitats and whether there are seasonal variations in circadian patterns. We believe that this kind of hypothesis is easy to test and would improve our knowledge of the ecology of Isospora spp. in natural populations of hosts.

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