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# Oocyst shedding by green-winged-saltator (*Saltator similis*) in the diagnostic of coccidiosis and *Isoospora similis* n. sp. (Apicomplexa: Eimeriidae)

Eliminação de oocistos por trinca-ferro-verdadeiro (*Saltator similis*) no diagnóstico da coccidiose e *Isoospora similis* n. sp. (Apicomplexa: Eimeriidae)

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## Abstract

Diurnal periodicity is a phenomenon that has been observed in coccidian of *Isoospora* parasites of passerines, which have been eliminated great number of oocysts at dusk. The objective of this study was to evaluate the occurrence of periodicity of oocysts presence in the green-winged-saltator *Saltator similis*, and its use in the diagnosis of coccidiosis in wild birds in captivity. A total of 220 fecal samples were collected from birds, apprehended from illegal trading and kept in quarantine in CETAS/IBAMA, in the morning and late afternoon, from May to November 2010. It was observed that 1.82% of the samples collected in the morning were positive, while 31.36% of samples were positive in the late afternoon. In addition, the number of oocysts shed was greater in the afternoon. Therefore, it was concluded that the sampling in the late afternoon provided greater reliability for the diagnosis of coccidiosis in green-winged-saltators. Moreover, in this study a new isosporoid coccidian parasite from the green-winged-saltator *S. similis* was observed and is herein described. *Isoospora similis* n. sp. oocysts are spheroidal to sub-spheroidal,  $27.5 \times 25.9 \mu\text{m}$ , with a smooth and bi-layered wall,  $\sim 1.2 \mu\text{m}$ . Micropyle and oocyst residuum are absent, but splinter-like or comma-like granules are present. Sporocysts are ellipsoidal or slightly ovoidal,  $17.4 \times 12.2 \mu\text{m}$ . A stieda body and substieda body are present. The sporocyst residuum is composed of granules of different sizes. Sporozoites are vermiform with a single refractile body and a nucleus. This is the fourth description of an isosporoid coccidium infecting *S. similis* and the sixth description from Cardinalidae.

**Keywords:** Periodicity, coccidian, oocysts, morphology, diagnosis.

## Resumo

A periodicidade diurna é um fenômeno que tem sido observado em coccídios do gênero *Isoospora* parasitas de pássaros, os quais eliminam uma maior quantidade de oocistos ao entardecer. O objetivo deste estudo foi determinar a periodicidade de eliminação de oocistos pelas fezes no trinca-ferro-verdadeiro *Saltator similis*, e sua utilização no diagnóstico da coccidiose. Foram colhidas 220 amostras fecais de aves oriundas de apreensões do tráfico de animais silvestres e mantidas na quarentena do CETAS/IBAMA, nos períodos da manhã e ao entardecer, de maio a novembro de 2010. Observou-se que 1,82% das amostras colhidas no período da manhã foram positivas, enquanto que 31,36% das amostras colhidas foram positivas ao entardecer, onde o maior número de oocistos foi observado no período da tarde. Portanto, concluiu-se que a colheita de amostras ao entardecer oferece maior confiabilidade para o diagnóstico da coccidiose. Além disso, descreve-se um novo coccídio do trinca-ferro-verdadeiro *S. similis*. *Isoospora similis* n. sp. possui oocistos esféricos a subesféricos,  $27,5 \times 25,9 \mu\text{m}$ , com parede dupla e lisa,  $\sim 1,2 \mu\text{m}$ . A micrópila e o resíduo do oocisto estão ausentes, porém pequenos grânulos estão presentes. Os esporocistos são elipsóides ou levemente ovóides,  $17,4 \times 12,2 \mu\text{m}$ . Os corpos de Stieda e substieda estão presentes. O resíduo do esporocisto está presente e os esporozoítos possuem um corpo refrátil posterior e um núcleo. Esta é a quarta espécie isosporóide descrita de *S. similis* e a sexta descrição na família Cardinalidae.

**Palavras-chave:** Periodicidade, coccídios, oocistos, morfologia, diagnóstico.

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## Introduction

The green-winged saltator *Saltator similis* Lafresnaye d'Orbigny, 1837 is a cardinalid bird resident in Brazil. Its natural populations have been decimated by illegal mining and trade, because of its beauty and vocal repertoire. Among the information available on this species, it is known that it does not present sexual dimorphism and its locomotion is mainly by jumping (SICK, 1997, IUCN, 2011).

Estimation of the intensity of coccidial infection is essential for studies of the impact of parasitism upon natural populations (DOLNIK, 2006). The parasite-host relationship in the species of birds, which has been the focus of research over the past decade, has great relevance to coccidian intestinal parasites, which parasitize the majority of vertebrate species and are closely associated with the ecology of birds (LÓPEZ et al., 2007). The phenomenon of diurnal periodicity has been observed in species of intestinal parasites, especially the genus *Isospora* Schneider, 1881 (Protozoa: Apicomplexa) in wild birds, which eliminated their oocysts more frequently in the late afternoon (BOUGHTON, 1937; DOLNIK, 1999, 2006; BROWN et al., 2001; LÓPEZ et al., 2007; MARTINAUD et al., 2009). Many studies on the periodicity of elimination of oocysts have been conducted in several species of passerines such as: blackcap *Sylvia atricapilla* L., 1758; island canary *Serinus canaria* L., 1758; garden warbler *Sylvia borin* Boddaert, 1783; Eurasian blackbird *Turdus merula* L., 1758; European greenfinch *Carduelis chloris* L., 1758; house finch *Carpodacus mexicanus* Müller, 1776; small ground-finch *Geospiza fuliginosa* Gould, 1837; regent honeyeater *Xanthomyza phrygia* Shaw, 1794; and house sparrow *Passer domesticus* L., 1758 (BOX, 1977; BRAWNER III; HILL, 1999; BROWN et al., 2001; DOLNIK, 2006; LÓPEZ et al., 2007; LINDSTROM et al., 2009; MARTINAUD et al., 2009; DOLNIK et al., 2010; MORIN-ADELINE et al., 2011; PAP et al., 2011).

However, in species of Brazilian passerines, there are no reports regarding diurnal periodicity in studies of prevalence or for diagnosis of coccidiosis in free-living wild birds or birds kept in captivity. Silva et al. (2010) reported that during periods of reproduction and moulting, wild birds kept in captivity were more prone to infection by *Isospora* spp.

This study aimed to determine the periodicity of oocysts shedding and identify new species of *Isospora*, not yet described in the scientific literature, from green-winged saltators *S. similis* recovered from the illegal trade of wild animals, which were kept under quarantine in preparation for release.

## Materials and Methods

In a total of 220 fecal samples were obtained from 164 green-winged saltators *S. similis* recovered from the trafficking of wild animals and kept in quarantine in CETAS (Centro de Triagem de Animais Silvestres - Center for Triage of Wild Animals)/IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis - Brazilian Institute of Environment and Natural Resources)/MMA (Ministério do Meio Ambiente - Ministry of Environment) (22° 43' 23.79" S and 43° 42' 36.94" W),

which is located at the Municipality of Seropédica in the State of Rio de Janeiro, Brazil. The collection period was from May to November 2010.

Two hundred and twenty samples were collected according to Dolnik (2006) and Dolnik et al. (2009); fresh samples of feces, shed by a bird, were collected individually from a sheet of paper towel placed on the bottom of each cage, after cleaning in the morning (9-12h) and at late afternoon (15-17h). The collected samples were placed into plastic vials containing a 2.5% (w/v) solution of  $K_2Cr_2O_7$  at a ratio of 1:6 (v/v). Samples were sent to the Coccidia and Coccidiosis Laboratory at Universidade Federal Rural do Rio de Janeiro (UFRRJ) for analysis. Upon receipt they were placed in a thin layer (~5 mm) of  $K_2Cr_2O_7$  2.5% solution in Petri plates, with incubation at 23-28 °C for 10 days or until 70% of oocysts had sporulated. Oocysts were recovered by flotation in Sheather's sugar solution (S.G. 1.20) and were examined microscopically using the technique described by Duszynski and Wilber (1997).

The quantification of oocysts was conducted according by Dolnik (2006) and Dolnik et al. (2010), where the whole surface of the coverslip was observed to avoid counting errors which may be caused by the agglomeration of oocysts in some unobserved field. The results were expressed as oocysts per droplet (OoPD).

An additional 108 samples were used to determine each the periodicity of shedding of each *Isospora* species. 54 samples were collected in the morning, between 9:00-12:00 PM, and 54 samples was collected in the late afternoon (3:00-5:00 PM). In order to describe the fourth (novel) species, sporulated oocysts were obtained from samples collected in late afternoon.

Morphological observations (micropyle [M], oocyst residuum [OR], polar granule [PG], Stieda body [SB], substieda body [SSB], parastieda body [PSB], sporocyst residuum [SR], sporozoite [SZ], refractile body [SRB], nucleus [N]) and measurements, in µm, were performed using a binocular microscope Carl Zeiss with apochromatic oil immersion objective lens and ocular micrometer (K-15X PZO, Poland). Line drawings were prepared using a binocular microscope Wild M-20 with drawing tube. Pictures were taken using a digital camera (Model CD Mavica MVC-CD250 Sony®). Size ranges are shown in parenthesis followed by average and shape index (L/W ratio).

Statistical analysis by Fisher's test and Wilcoxon's test were performed using Excel XP (Microsoft Co., Redmond, WA, USA), as proposed by Sampaio (2002).

## Results

Seventy-three of 220 samples examined in this study for evaluation of the incidence of oocysts shedding, were positive for *Isospora* spp. Only 1.82% of the samples collected in the morning had positive results. In the late afternoon, 31.36% of the samples were positive (Table 1). Furthermore, oocyst shedding was quantitatively greater in the late afternoon, with a mean value of 98 OoPD, in comparison with the mean value of <1 OoPD recorded for the morning period (Table 2). These results were highly significant ( $p = 0.0001$ ).

All of the sporulated oocysts examined were typical of the genus *Isospora*. Four morphotypes of oocysts were recorded. The first three

**Table 1.** Fecal samples of green-winged saltators, *Saltator similis*, used in the evaluation of the incidence of oocyst shedding.

Period <sup>a</sup>	Presence of oocysts		Total	P value <sup>b</sup>
Morning	Positive	4 ( 1.82 %)	110 (50%)	0.0001
	Negative	106 (48.18 %)		
Late afternoon	Positive	69 (31.36 %)	110 (50%)	
	Negative	41 (18.64 %)		
Total			220 (100%)	

<sup>a</sup>Sampling in the period of the morning, between 9:00-12:00 AM, and late afternoon, between 3:00-5:00 PM. <sup>b</sup>Highly significant by Fisher's test.

**Table 2.** Distribution of the mean values of number of oocysts in the fecal samples of green-winged saltators, *Saltator similis*, by period.

Period <sup>a</sup>	OoPD <sup>b</sup>	P value <sup>c</sup>
Morning	N	110
	Mean value	0.04 (0.0-1.0)
Late afternoon	N	110
	Mean value	98.3 (0.0-3668)

<sup>a</sup>Sampling in the period of the morning, between 9:00-12:00 AM, and late afternoon, between 3:00-5:00 PM. <sup>b</sup>Number of oocysts per droplet. <sup>c</sup>Highly significant by Wilcoxon's test.

types were identified as: (1) *Iso spora vanriperorum* Levine, 1982; (2) *Iso spora saltatori* Berto et al., 2008; (3) and *Iso spora trincaferri* Berto et al., 2008. The fourth type of oocysts was different from the above cited species and was considered to represent an unknown species, the description of which is provided below.

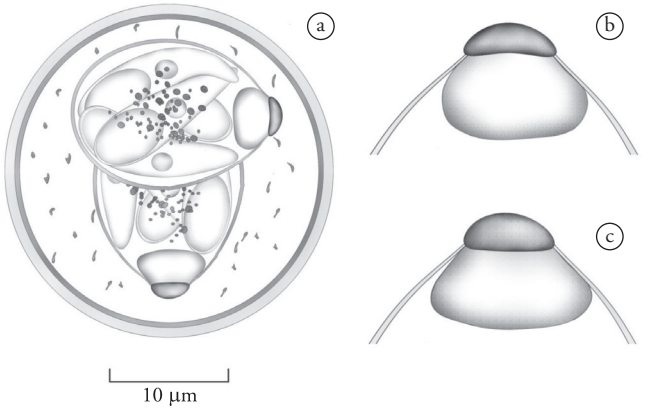
*Iso spora similisi* n. sp. (Figures 1a-c, 2a, b)

**Description of a sporulated oocyst:** Oocyst shape (N = 25): spheroidal to sub-spheroidal; number of walls: 2; wall thickness: 1.2 (1.1-1.3); smooth outer wall about 2/3 of total thickness; L x W: 27.5 x 25.9 (26-29 x 24-28), with L/W ratio: 1.1 (1.0-1.1); M and OR: absent; PG: present, forming splinter-like or comma-like granules.

**Description of a sporocyst and sporozoites:** Sporocyst shape (N = 25): ellipsoidal or slightly ovoidal; L x W: 17.4 x 12.2 (15-19 x 11-13); L/W ratio: 1.4 (1.2-1.7); SB: present, knob-like, 1.4 high x 2.6 wide; SSB: present, large, homogeneous, 2.6 high x 4.5 wide; PSB: absent; SR: present; SR characteristics: composed of granules of different sizes; SZ: vermiform with 1 posterior SRB and centrally located N.

**Type host:** *Saltator similis* Lafresnaye d'Orbigny, 1837 (Aves: Passeriformes: Cardinalidae).

**Type material:** One-half of the oocysts are kept in 10% aqueous buffered formalin (v/v) and the other half in 70% ethanol according Duszynski and Gardner (1991). Both samples were deposited in the Parasitology Collection, in the Department of Animal Parasitology, at UFRRJ, located in Seropédica, Rio de



**Figure 1.** Line drawings of *Iso spora similisi* n. sp, a new coccidium species recovered from the green-winged saltator *Saltator similis*. (a) Sporulated oocyst with its respective variations of (b, c) detached Stieda and substieda bodies. Scale-bar: 10 µm for oocysts; and 5 µm for Stieda and substieda bodies.

Janeiro, Brazil. Phototypes and line drawings are deposited as well. The repository number is P-41/2011.

**Type locality:** Seropédica. (22° 43' 23.79" S and 43° 42' 36.94" W), Rio de Janeiro, Brazil.

**Sporulation time:** seven days.

**Site of infection:** Not investigated.

**Prevalence:** 59% (32 of 54 birds examined).

**Etymology:** The specific epithet is derived from the specific name of the type host.

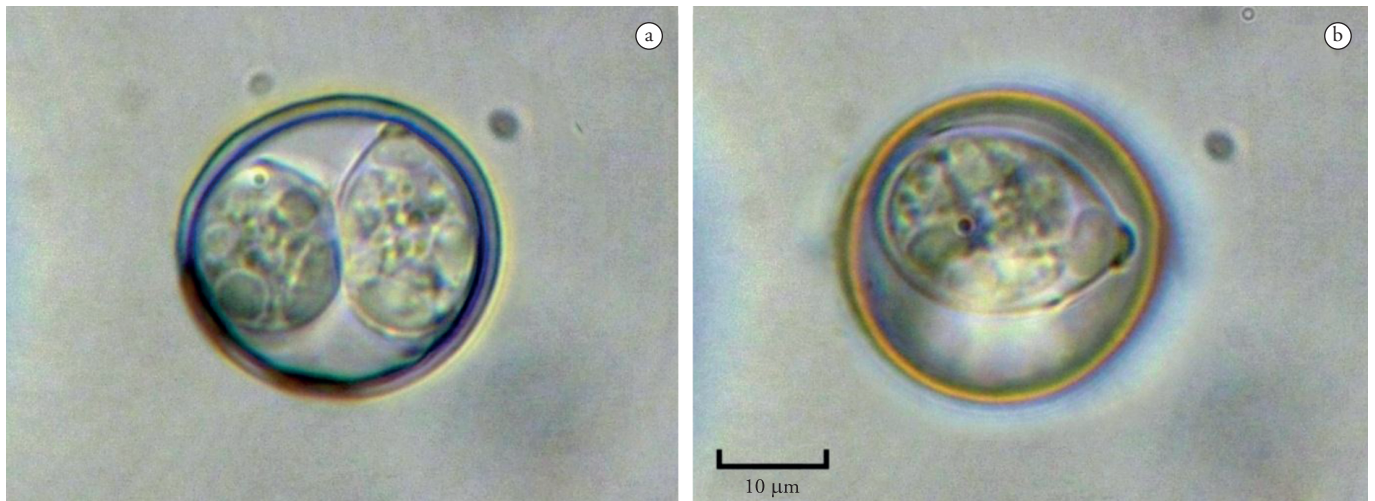
**Comments:** *Iso spora similisi* differs from other *Iso spora* spp. from the same host family (Table 3). Only *I. trincaferri* had similar dimensions with *I. similisi*; however, it can be distinguished by the unique PG and bubble-shaped SB (BERTO et al., 2008, 2011b).

The periodicity associated with *Iso spora* spp. is presented in the Table 4. Firstly, a larger number of oocysts were recovered in the late afternoon. Secondly, in the late afternoon, four *Iso spora* spp. were indentified (*I. trincaferri*; *I. vanriperorum*; *I. saltatori*; and *I. similisi*), in contrast, in the morning only *I. trincaferri* and *I. vanriperorum* were indentified. *Iso spora similisi* showed the highest mean of OoPD in the late afternoon; and *I. trincaferri* and *I. vanriperorum* have equal mean values of OoPD in the morning.

Discussion

This study determined the most probable period of oocyst shedding, which should result in more reliable diagnosis of coccidian infection during quarantine at a center for wild animals, where the passerines had suffered constant stress since their capture until their seizure by IBAMA. During winter in Rio de Janeiro, Brazil, the sunset happens earlier, between 5:00-6:00 PM and the samples used were collected in autumn, winter and spring. The period of sampling occurred in the morning, between 9:00-12:00 PM, and in late afternoon, between 3:00-5:00 PM, which covered the main times for treatment and management of animal sheld at CETAS. According to Brawnner III and Hill (1999), and Villanúa et al.





**Figure 2.** Photographs (a-b) of sporulated oocysts of *Isoospora similis* n. sp, a new coccidium species recovered from the green-winged saltator *Saltator similis*. Scale-bar: 10 µm.

(2006), the time of day is a crucial factor that must be taken into consideration when undertaking the sampling of bird droppings. The majority of species of coccidia that infect passerines belong to the genus *Isoospora* and during their developmental cycle, which includes oocyst shedding, there is a diurnal periodicity (BOUGHTON, 1937; DOLNIK, 2006).

In the present study only 1.82% of the samples collected in the morning were positive for coccidia but in the late afternoon, 31.36% of the samples were positive. Thus, these results are similar to those of Boughton (1933, 1937, 1988), Dolnik (1999, 2006), Brown et al. (2001), López et al. (2007), Lindstrom et al. (2009), Martinaud et al. (2009), Filipiak et al. (2009), Dolnik et al. (2010), Morin-Adeline et al. (2011) and Pap et al. (2011), where, in spite of different latitudes and longitudes, it was reported that the late afternoon was the most reliable period for sampling because it is the period during which oocyst shedding is greatest. In birds kept in captivity, the oocysts shedding begin earlier and ended later, when compared with the free-living passerines which have peak of oocysts shedding between 1:00-9:00 PM. Moreover, the number of oocysts shed during the first days of captivity was reported to be significantly higher than during longer times post capture (DOLNIK, 1999). Based on that report, the sampling in the current study was performed, whenever possible, on the first day of arrival of passerines in quarantine, with the objective of ensuring a more reliable diagnosis. In a study with house sparrows *P. domesticus* was reported that the periodicity of oocysts shedding was not associated with feeding; however, it was associated with photoperiod, because after the reversal of light and darkness, there is a reversal in the time of oocyst shedding. On other hand, it is known that oocyst shedding is controlled by physiology of the host and physiological differences between dietary habits are probably responsible for these differences (LÓPEZ et al., 2007). In the wild, birds show two peaks of feeding activity, one in the morning and another in the late afternoon. During this period, the birds feed in the same areas. In the breeding season, they feed on the same territory all day and, during the migration season, many species

gather in flocks to feed. Clearly, the presence of parasites in feces during this period would increase the chances of infecting new hosts (DOLNIK, 1999; MARTINAUD et al., 2009). It is of value to note, that, although the oocysts are relatively resistant to environmental factors, such as temperature and relative humidity, it has been reported that desiccation can reduce the infectivity of oocysts of *Eimeria* spp. in poultry production; this lack of infectivity was confirmed in an experiment with *Isoospora* infection in Eurasian blackbirds *T. merula*. It was observed that the release of oocysts in the late afternoon represented an adaptation to provide resistance to desiccation and ultraviolet radiation, because if oocysts were released throughout the day, most would be quickly destroyed by the action of sunlight (MARTINAUD et al., 2009). In some species of birds which begin to migrate, the feeding peak disappears in the morning, but the feeding peak at late afternoon never disappears. Thus, these birds gather with others, at least in a period of day, increasing the chances of infection of new hosts (DOLNIK, 1999).

Finally, we conclude that estimations of the prevalence and parasite load performed without considering the time of day are not reliable. A good method for accuracy would be to restrict the period of sampling. Collection of samples for quantification of parasite load for coccidia would be restricted to the second half of the late afternoon, while those intended for prevalence studies could be carried out between 1/2 and 4/5 of the late afternoon as suggested by López et al. (2007). The results of the current study were similar, because the sampling was restricted to the late afternoon, specifically between 3:00-5:00 PM. The oocysts' shedding was quantitatively greater in the late afternoon, with a mean value of 98 OoPD, in comparison with the mean value of <1 OoPD in the morning period, even in wild passerines kept in a quarantine regime.

The illegal animal trade had led to the reduction and extinction of some bird species worldwide. In addition, another consequence is the introduction of new parasites, as occurred with *Isoospora vanriperorum* Levine, 1982, first described from the northern

**Table 3.** Comparative morphology of *Isoospora similisi* n. sp and *Isoospora* spp. recorded from cardinalid birds.

Coccidia	Host(s)	Reference(s)	Oocysts			Sporocysts						
			Shape	Measurements (µm)	Shape index	Wall (µm)	Polar granule	Shape	Measurements (µm)	Stieda body	Substieda body	Residium
<i>Isoospora vanripiperorum</i> Levine, 1982	<i>Cardinalis cardinalis</i> Linnaeus, 1758; <i>Saltator similis</i> Lafresnaye d'Orbigny, 1837	Levine (1980) and Levine et al. (1982) Lopes et al. (2007)	sub-spherical	24 × 23 (22-26 × 20-25)	-	one-layered, c.0.8	present	ovoid	16 × 10 (15-17 × 10)	knob-like	indistinct	compact
<i>Isoospora pityli</i> McQuiston & Capparella, 1992	<i>Saltator grossus</i> <i>saturatus</i> Todd, 1922	McQuiston and Capparella (1992)	sub-spherical	20.1 × 18.8 (20-21 × 17-20)	1.1 (1.0-1.2)	bi-layered, c.1.5	absent	ovoid	14.7 × 9.4 (12-17 × 8-11)	nipple-like	absent	compact
<i>Isoospora formicarium</i> McQuiston & Capparella, 1992	<i>grossus grossus</i> Linnaeus, 1766; <i>S. g. saturatus</i>	McQuiston and Capparella (1992)	sub-spherical	24.6 × 23.5 (21-27 × 20-25)	1.0 (1.0-1.1)	bi-layered, c.1.5	absent	ovoid	15.7 × 11.3 (14-17 × 10-13)	small, nipple-like	large, triangular or conical	compact
<i>Isoospora saltatori</i> Berto, Balthazar, Flausino & Lopes, 2008	<i>S. similis</i>	Berto et al. (2008)	sub-spherical	18.3 × 17.9 (17-20 × 16-20)	1.0 (1.0-1.1)	bi-layered, c.1.1	absent	ovoid	13.4 × 8.9 (12-15 × 8-10)	small, flattened	small	compact
<i>Isoospora trincaferri</i> Berto, Balthazar, Flausino & Lopes, 2008	<i>S. similis</i>	Berto et al. (2008)	sub-spherical	26.2 × 23.6 (24-29 × 22-25)	1.1 (1.0-1.2)	bi-layered, c.1.2	present	ovoid	17.5 × 11.5 (17-18 × 10-13)	bubble-shaped	prominent	diffuse
<i>Isoospora similisi</i> n. sp.	<i>S. similis</i>	current study	spherical to sub-spherical	27.5 × 25.9 (26-29 × 24-28)	1.1 (1.0-1.1)	bi-layered, c.1.2	present, splinter-like or comma-like granules	ellipsoidal or slightly ovoidal	17.4 × 12.2 (15-19 × 11-13)	knob-like	large, homogeneous	diffuse

**Table 4.** Periodicity of shedding *Isoospora* oocysts from green-winged saltators *Saltator similis* at CETAS/IBAMA, Seropédica in the State of Rio de Janeiro, Brazil.

Species	Morning <sup>a</sup> (n = 54)		Late afternoon <sup>b</sup> (n = 54)	
	OoPD <sup>c</sup>	Means	OoPD <sup>c</sup>	Means
<i>I. trincaferri</i>	2	1	894	59.60
<i>I. vanriperorum</i>	2	1	1088	83.69
<i>I. saltatori</i>	0	0	1952	48.80
<i>I. similis</i>	0	0	5789	175.42
Total	4	0.074	9723	180.05

<sup>a</sup>Sampling in the period of the morning, between 9:00-12:00 AM. <sup>b</sup>Sampling in the late afternoon, between 3:00-5:00 PM. <sup>c</sup>Number of oocysts per droplet.

cardinal *Cardinalis cardinalis* L., 1758, but which due to its introduction into South America for breeding in captivity, was subsequently reported from *S. similis* (LOPES et al., 2007; BERTO et al., 2011b).

The ease of transmission of *Isoospora* among birds from the same family, the large number of cardinalid species in New World and the illegal bird trade may serve to ensure a wide distribution of these parasites in South, Central and North America (BERTO et al., 2011b).

In Brazil, the native birds are protected by law and supervised by the Brazilian Institute of the Environment and Natural Renewable Resources (IBAMA), therefore a symbiotype could not be collected (IBAMA, 2011).

According to Duszynski and Wilber (1997) and Berto et al. (2011b), a new coccidian species should be morphologically compared with other congeneric coccidian species, that share similar features and belong to the same host family. Thus, *I. similis* sporulated oocysts was compared to all of described species from Cardinalidae.

As observed in the current study, a few variations in SB and SSB (Figure 1b, c) were also observed in sporocysts of *I. similis* according to Grulet et al. (1982), Berto et al. (2009a, b, c, 2010, 2011a, c), Coelho et al. (2011a, b) and Pereira et al. (2011); however, these variations are not significant enough to separate and describe it as new species. These variations could possibly be the result of the sporulation process, the position of the SZ inside of the sporocyst, or the position of the oocyst and sporocyst under the coverslip.

Based upon its morphological features, *I. similis* is considered as new to science, being the fourth description from *S. similis* and the sixth description from the host family Cardinalidae. Interestingly, based upon the OoPD values, this species was considered to be the most competitive among the four species of *Isoospora* associated with *S. similis*.

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