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Posted Date: 19 May 2025

doi: 10.20944/preprints202505.1489.v1

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Article

Weight Development of Intermediate Hosts Infected by *Cystoisospora ohioensis*-Like Species (Apicomplexa: Cystoisosporinae): Experimental Model in Mice

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Simple Summary: The study investigated the effects of *Cystoisospora ohioensis*-like infection in mice as intermediate hosts, focusing on weight development, visceral changes, and transmission to dogs. The problem addressed was the lack of information on the impacts of these parasites on intermediate hosts, particularly regarding weight gain and visceral health. The objectives included evaluating clinical signs, weight changes, and the presence of hypnozoites in the organs of infected mice, as well as testing the infectivity of these parasites in dogs. The results showed that infected mice had higher body weight and weight gain compared to the control group, but lower carcass yield due to increased organ weight. Hypnozoites were found in the intestines, lymph nodes, and spleen, persisting for up to 60 days. Dogs infected with hypnozoites had a shorter prepatent period and higher oocyst shedding. The conclusion emphasized that *C. ohioensis*-like affects intermediate host development, suggesting these animals should be classified as intermediate hosts rather than mere carriers. The study is valuable to society by clarifying the negative effects of these parasites on animal production and health, contributing to more effective control strategies in animals and potentially in humans, especially in immunosuppressed contexts.

Abstract: This study aimed to evaluate clinical signs, visceral changes, and weight development in intermediate hosts infected with *Cystoisospora ohioensis*-like oocysts from naturally infected dogs. Using 135 albino mice divided into three groups—INFECTED (inoculated with oocysts), PAIR-FED (given the same food as infected mice), and CONTROL (fed *ad libitum*)—researchers monitored weight and pathological changes over 35 days. Mice were euthanized at various intervals post-inoculation (1–35 days), with organs from some INFECTED mice fed to dogs on day 60 for a biological assay. The INFECTED group showed higher body weight and weight gain than the CONTROL group, though organ weights were greater, leading to lower carcass yield. Tissue cysts with hypnozoites were found in the intestines (1st day), Peyer's patches (up to 5th day), and lymph nodes/spleen (up to 35th day). Dogs inoculated with hypnozoites (from infected mice) had a shorter prepatent period, longer patent period, and higher oocyst shedding compared to those given sporulated oocysts. The findings indicate that *C. ohioensis*-like species affect mouse weight, persist in visceral tissues for up to 60 days, and exhibit biological differences in infectivity between sporozoites and hypnozoites in dogs.

Keywords: Cystoisosporosis; oocysts; hypnozoites; host infectivity

1. Introduction

The genus *Cystoisospora* was established by Frenkel [1] to accommodate certain species formerly classified under *Isospora*, characterized by sporulated oocysts containing two sporocysts with four sporozoites each, the absence of Stieda bodies in the sporocysts, and the ability to form monzoic tissue cysts (hypnozoites) in intermediate hosts. Smith [2], who recognized the involvement of an intermediate host, proposed placing *Cystoisospora* within the subfamily Cystoisosporinae under the family Sarcocystidae. Molecular studies subsequently confirmed the classification of mammalian *Cystoisospora* species within the Sarcocystidae family [3–6].

Within the subfamily Cystoisosporinae, the genus *Cystoisospora* comprises four species for which the dog is the definitive host: *C. canis*, *C. ohioensis*, *C. burrowsi*, and *C. rivolta*. Among these, *C. canis* is distinct because of its large, ovoid oocysts (28–34 μm), whereas the latter three, with intermediate-sized oocysts (17–23 μm), are considered *C. ohioensis*-like owing to their overlapping size ranges [7,8]. The third group, consisting of small oocysts (10–13 μm) shed in the feces of infected dogs, includes two members of the subfamily Toxoplasmatinae: *Neospora caninum*, which is clinically significant, and *Heydornia heydorni* (syn. *Hammondia heydornii*), which is nonpathogenic [9]. The developmental stages of these parasites are primarily found in the digestive tracts of young animals, which become infected by ingesting sporulated oocysts from the environment or monzoic cysts present in the viscera of previously infected intermediate hosts [10]. *Cystoisospora ohioensis* has been characterized as pathogenic when experimentally inoculated into puppies, which subsequently develop diarrhea as a clinical manifestation [11,12]. Additionally, diarrhea associated with *C. ohioensis* has been reported in a naturally infected three-month-old puppy in South Korea, with the diagnosis confirmed by PCR [13].

Cystoisospora ohioensis is widely distributed and fairly common in epithelial cells throughout the small intestine as well as in the cecum and colon of dogs, coyotes, red foxes, raccoons, and possibly dingoes [14]. Dogs fed tissues from camels [15,16], sheep [15], pigs, donkeys, and buffaloes [17] naturally shed *C. ohioensis* oocysts in their feces; however, there has been no microscopic confirmation of monzoic tissue cysts. Experimentally, intermediate hosts include mice, rats, golden hamsters, cats, and dogs, in which monzoic cysts have been found in viscera, mainly mesenteric lymph nodes, liver, and spleen [14]. Frenkel and Dubey [18], in describing mice as intermediate hosts of *Cystoisospora* species in felines, characterized the formation of merozoites through endodyogeny as an asexual reproductive process occurring in the mucosa of the small intestine of intermediate hosts. Subsequent studies led Dubey and Lindsay [8] to suggest that the two zoites likely enter the same host cell rather than resulting from the division of a single zoite. The monzoic tissue cysts of *C. felis*, *C. rivolta*, and *C. ohioensis* increase in size over time but do not undergo division [10,18]. Thus, the formation of monzoic cysts is considered one of the distinguishing characteristics of *Cystoisospora* species [1], a feature also observed in the asexual stages of *Cystoisospora* spp. in mouse tissues [19].

There is limited information regarding the economic significance of species within this genus, although the presence of the parasite has been reported in the tissues of naturally infected livestock [15,17] as well as in experimentally infected livestock [20–23]. Pathologies associated with monzoic cysts have been reported for the genus *Cystoisospora* in humans, particularly in cases involving *Cystoisospora belli*, the causative agent of human cystoisosporosis [24]. This organism has been identified as an opportunistic etiological agent in patients with acquired immunodeficiency syndrome (AIDS) and has been associated with chronic diarrhea, acalculous cholecystitis, and cholangiopathy [25,26]. Velásquez et al. [27] reported that in this patient group, infection by *C. belli* may lead to disseminated cystoisosporosis, with the presence of monzoic tissue cysts in the intestinal lamina propria, lymph nodes, liver, and spleen [28–32].

To diagnose coccidiosis in dogs, it is necessary to detect oocysts through microscopic examination [14] or identify the developmental stages of the parasite in mucosal scrapings during the acute phase of infection [33,34]. Lee et al. [13] examined the feces of a three-month-old female dog with persistent diarrhea using real-time multiplex polymerase chain reaction (PCR), which targeted 23 pathogens known to cause diarrheal syndromes. Sequence analysis was performed using nested

PCR amplification of 18S ribosomal RNA. Oocysts were identified in the fecal smear. Although real-time multiplex PCR was positive for *Cyclospora cayetanensis*, the final diagnosis was *C. ohioensis* infection, which was confirmed through phylogenetic analysis of the 18S rRNA gene. Another relevant aspect is the difficulty in isolating oocysts of *C. canis* [1,35] and *C. ohioensis* [1,36], as these species are most often shed in the feces of dogs along with other coccidia, such as *C. burrowsi* [37] Rommel & Zielasko, 1981 [38], *C. rivolta* [39] Frenkel, 1977 [1], as well as small oocysts of *Heydornia heydorni* (syn. *Hammondia heydorni*) [40], *Neospora caninum* [41], and some species of the genus *Sarcocystis*.

Thus, from the diagnosis of the shedding of subspherical, disporic, tetrazoic coccidian oocysts of medium size by six dogs from the same litter—morphologically and morphometrically characterized as belonging to the *C. ohioensis*-like group—the objective of this research was to experimentally infect mice, describe possible clinical signs of cystoisosporosis in these animals, and evaluate whether there are alterations in the viscera of the infected animals and whether cystoisosporosis influences the weight development of the intermediate host using an experimental mouse model.

2. Materials and Methods

This study is part of a doctoral thesis [42] and was conducted at the Protozoology Laboratory of the Experimental Station for Parasitological Research under Dr. Wilhelm Otto Neitz, the Department of Animal Parasitology, the Institute of Veterinary Medicine, the Federal Rural University of Rio de Janeiro (UFRRJ), Seropédica Campus, RJ, Brazil.

2.1. Origin of the Oocysts

Cystoisospora ohioensis-like oocysts were obtained from the feces of six eight-week-old puppies from the same litter. The animals were naturally infected with *Cystoisospora* spp. and lived freely in peridomestic urban areas of the city of Seropédica, State of Rio de Janeiro, Brazil. They were maintained in accordance with Law No. 6,638 of May 8, 1979, repealed by Law No. 11,794/2008, which regulates the licensing of activities related to the production, maintenance, or use of animals for education or scientific research in Brazil [43], and in accordance with CFMV Resolution No. 714/2002, which establishes authorization requirements for animal experimentation and requires that the method chosen must depend on the species involved, the available means for animal restraint, the technical ability of the practitioner, and the number of animals [44]. The animals were fed a commercial diet formulated for puppies provided *ad libitum*. Pens were cleaned daily and sanitized weekly using a flame broom. For 15 days, the feces were examined daily for oocyst detection and collection, according to Oliveira et al. [43].

2.2. Oocyst Sporulation

The collected fecal material was diluted at a ratio of 1:2 in a 2.5% potassium dichromate solution and aerated using a conventional aquarium pump (Alpha II, Vigor Ar, São Paulo) at room temperature. After 72 hours of aeration, a drop of the suspension was placed between a microscope slide and coverslip and examined under a light microscope. Once the sporulation index reached 80% or higher, the fecal suspensions were transferred to 50 mL Falcon-type centrifuge tubes and centrifuged at $200 \times g$ for 10 minutes. The sporulated oocysts were recovered from the supernatant using flotation in a saturated sugar solution.

2.3. Oocyst Concentration

The sporulated oocysts were concentrated by resuspension in a saturated sugar solution followed by additional centrifugation ($200 \times g$ for 5 minutes). The resulting supernatant was poured into a 14 cm diameter Petri dish, over which the bottom of a 15 cm Petri dish was placed, creating an interface between the lid (the base of the 15 cm Petri dish) and the convergent meniscus of the sugar solution surface. At 15-minute intervals over one hour, the upper dish was removed so that its bottom

could be rinsed with phosphate-buffered saline (PBS), pH 7.2, using a 10 mL syringe fitted with a hypodermic needle. The suspension of sporulated oocysts obtained from washing with PBS was collected using a funnel placed in a glass beaker. These oocysts were subjected to another round of centrifugation, followed by two consecutive resuspensions in PBS to eliminate potassium dichromate residue and excess sucrose. The final sediment, containing a high concentration of oocysts, was resuspended in a small volume of PBS and transferred to a new sterile 50 mL Falcon tube. The total volume of feces excreted by the dogs was concentrated to a final volume of 50 mL and stored at 4 °C until use.

2.4. Oocyst Counting and Morphometry

An aliquot of purified oocyst suspension was placed in a Neubauer chamber for counting. The average number from five counts of oocysts quantified in the four outer reticulated areas—the area used for leukocyte quantification—was multiplied by 2.5, which is the chamber height correction factor, corresponding to the number present in 1 mm³ of the material. To convert the number of oocysts per mL of the concentrated suspension, the number of oocysts found in 1 mm³ was multiplied by 1,000. Thus, the total number of oocysts recovered from all the samples collected from all the animals was equal to the number of oocysts quantified in one mL multiplied by the total volume of the concentrated suspension. The major diameter (MD) and minor diameter (md) of sporulated oocysts were measured one hundred times via a K-15X micrometer eyepiece (PZO, Poland) coupled with a Leitz Westler microscope (H.M. Lux, Germany). The morphometric indices (MIs) were obtained by the ratio of MD to md and were calculated for both sporulated oocysts and the sporocysts within each oocyst.

2.5. Standardization of the Inoculum and Infection of the Mice

For the concentrated sample, the total number of oocysts in the sample was previously evaluated, and the inoculum was standardized for each mouse to 10⁵ sporulated oocysts of the *C. ohioensis*-like group in 0.5 mL of PBS.

A total of 135 albino mice from the Central Animal Facility of Fundação Instituto Oswaldo Cruz (FIOCRUZ) were distributed into 27 polypropylene cages with five animals per cage. These animals were divided into three groups with nine cages and 45 mice per group. In one of the groups, the mice received the standardized inoculum orally using a gavage needle. This group was named INFECTED, and the animals were fed species-specific feed *ad libitum*. The other two groups, with a total of 90 mice, received 0.5 mL of PBS as the inoculum and served as experimental controls. One of these groups with 45 mice was fed *ad libitum* and called CONTROL, whereas the other 45 mice received, throughout the entire experiment, the same amount of feed consumed by their counterparts in the INFECTED group and was named the pair-fed group (PAIR FED). All 135 mice in the INFECTED, CONTROL and PAIR FED groups received water *ad libitum* throughout the experiment and were maintained in compliance with Law No. 6,638 of May 8, 1979 (revoked by Law No. 11,794/2008 – [44]), in accordance with CFMV Resolution No. 714/2002 [45].

2.6. Evaluation of Body Weight Development

The relative weights of the mice, whether infected or not, were proportionally assessed through daily weight measurements of each animal over 35 days. The influence of food intake on weight was evaluated by comparing the mean body weight of the infected group with that of the control groups.

Five mice from each group were euthanized in a CO₂ chamber at 1, 3, 5, 9, 14, 21, 28, 35, and 60 days after infection (DAI), in accordance with the subsequently established Law No. 11,794/2008 (which revoked Law No. 6,638 of May 8, 1979; [44]) and CFMV Resolution No. 714/2002 [45]. Animals euthanized between 1 and 35 DAI underwent necropsy and evisceration. Their organs—stomach, small intestine, large intestine, Peyer's patches, mesenteric lymph nodes, spleen, liver, lungs, and heart—were collected, identified, and weighed using a precision balance (Mettler PE 360, USA). For each subject, the proportional weight of the viscera relative to the live body weight was determined.

Similarly, the carcass weight postvisceration was comparatively assessed. The organs of the mice euthanized at 60 DAI were collected for subsequent hypnozoite recovery.

2.7. Recovery of Hypnozoites from Organs

For each of the five mice euthanized at 60 DPI, their organs were collected, weighed, and individually minced into small fragments using a mortar and pestle. A 0.9% saline solution was then added to achieve a final homogenate volume of 5 mL, which was transferred to a 50 mL plastic centrifuge tube. *Cystoisospora ohioensis*-like hypnozoites were recovered according to the methodology described by Dubey [46], with slight modifications as proposed by Oliveira et al. [47].

2.8. Infectivity of Sporozoites and Hypnozoites

To determine the viability of inocula containing 10^5 sporulated oocysts of the *C. ohioensis*-like group and hypnozoites present in the viscera of mice from the INFECTED group, a pregnant bitch (in her final trimester) from Rio de Janeiro was selected and maintained at the UFRRJ experimental station. The bitch received daily oral treatment with trimethoprim (90 mg) and sulfadiazine (410 mg) (Triglobe®, Astra Química e Farmacêutica Ltda., Tamboré, São Paulo, Brazil) for 10 days and served as a puppy donor. Six puppies of both sexes born to this bitch were dewormed at 25 days of age (Basken® Plus, König do Brasil Ltda; single dose of 120 mg/kg body weight), weaned at 35 days, and housed in individual cages. Throughout the experiment, the animals received commercial puppy feed and water ad libitum. Two puppies were inoculated with 0.5 mL of a concentrated suspension containing 10^5 sporulated oocysts. Another two puppies were fed organs from INFECTED group mice (euthanized at 60 DAI). The remaining two puppies served as controls: one received 0.5 mL of PBS orally, while the other was fed homogenized organs from CONTROL and PAIR-FED group mice.

2.9. Recovery of Oocysts from the Biological Assay

Oocysts recovered from the feces of two dogs inoculated with sporulated oocysts and two dogs fed viscera from mice in the INFECTED group were quantified daily using a modified Sheather's sucrose flotation technique, with slight adaptations as follows: the total fecal matter excreted daily by the dogs was collected, weighed, and homogenized. A 1-gram aliquot was placed in a 125 mL Erlenmeyer flask containing 100 mL of distilled water. The material was mixed to obtain a homogeneous suspension, from which 10 mL was withdrawn and transferred to a 15 mL Falcon centrifuge tube. The sample was subsequently centrifuged at $200 \times g$ for five minutes. The supernatant was discarded, and the sediment was resuspended in saturated sugar solution before being centrifuged again under the same conditions ($200 \times g$ for 5 minutes). A disposable Pasteur pipette was used to add saturated sugar solution until a meniscus formed. A coverslip was placed on top for five minutes and then carefully transferred to a standard glass slide. The total number of oocysts present between the slide and coverslip was counted and multiplied by 10 to obtain the number of oocysts per gram of feces (OoPG). The total daily oocyst output was calculated by multiplying the OoPG value by the total weight (in grams) of feces collected each day. The remaining fecal material containing oocysts was further processed for concentration, sporulation, and morphological/morphometric analysis of the oocysts, following the same protocol used for oocysts collected from naturally infected dogs and initial donor animals in this study.

2.10. Statistical Analysis

The daily mean OoPG excreted and total daily output, along with the mean measurements and morphometric indices of oocysts and sporocysts from both naturally infected dogs and those inoculated with hypnozoites or oocysts, were subjected to descriptive analysis, and their means were compared using Student's *t* test. The mean values of live weight, weight gain, carcass weight, carcass yield, total visceral weight, percentage weight of each organ, and mean percentage organ weight relative to carcass weight from mice in the INFECTED, PAIR-FED and CONTROL groups were compared by Tukey's test. Additionally, the mean values of MD, md and MI of oocysts and

sporocysts excreted by naturally infected dogs, dogs experimentally infected with sporulated oocysts, and dogs fed viscera (hypnozoites) from mice infected with sporulated oocysts were calculated and compared by Tukey's test. The number and percentage of hypnozoites in the viscera of the mice that were fed sporulated oocysts were also calculated. For statistical analysis of the data, GraphPad InStat software was used (<https://graphpad-instat.software.informer.com/>).

3. Results

A total of 26.73×10^9 oocysts were recovered from the six naturally infected dogs over 15 days of shedding. None of the puppies presented clinical manifestations, and the texture of the collected feces was consistent in terms of their characteristic color and odor. The daily shedding behavior of the six puppies during the collection period can be observed in Figure 1a.

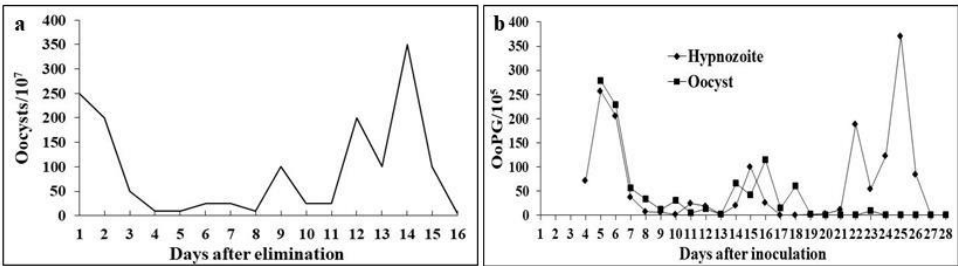


Figure 1. *Cystoisospora ohioensis*-like oocysts shed daily by dogs. a, Total oocysts excreted by six naturally infected 8-week-old puppies. b, Mean daily excretion of oocysts per gram of feces (OoPG) divided by 1,000 (OoPG/1,000) from two puppies inoculated with viscera from five mice previously infected with 10^5 sporulated oocysts of *C. ohioensis*-like species.

The sporulated *C. ohioensis*-like oocysts had a subspherical to spherical shape, with an oocyst wall consisting of two membranes averaging $0.29 \mu\text{m}$ in thickness and lacking a micropyle. The sporocysts were ellipsoidal in shape, with a smooth and thin wall enclosing four sporozoites inside, and no Stieda, Substieda, or Parastieda bodies were observed, nor were polar granules or oocyst residues. Sporocyst residues appeared as fine granular material that formed small clumps that aggregated into larger, denser granules (Figure 2). The mean dimensions and standard deviations of the MD, md and MI, as well as the highest observed values for oocysts and sporocysts, are shown in Table 1.

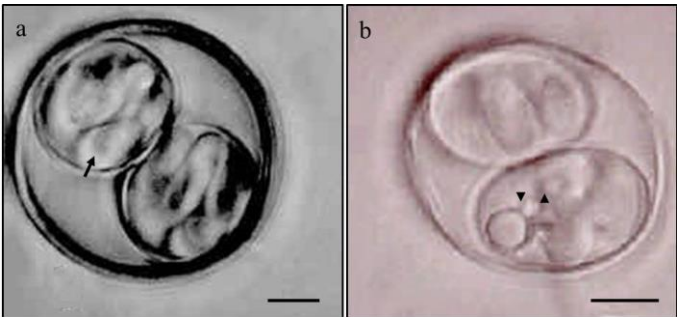


Figure 2. Photomicrograph of sporulated oocysts of *Cystoisospora ohioensis*-like species excreted in feces by (a) naturally infected 8-week-old dogs and (b) dogs experimentally infected with 10^5 sporulated oocysts at 35 days of age. Highlighted in (a) sporozoites (arrow) and (b) fine granular residual bodies (arrowhead). Scale bar: $5 \mu\text{m}$.

Table 1. Dimensions in micrometers of oocysts and sporocysts and morphometric indices of *Cystoisospora ohioensis*-like organisms, excreted by naturally infected dogs, experimentally infected with sporulated oocysts (sporozoites), and fed viscera (hypnozoites) from five mice infected with sporulated oocysts.

Route of Infection	n ^a	Diameters	Morphometric
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Measured forms		Major	Minus	Indice
Natural				
Oocyst	100	21.39±1.71 (18.40-26.80)	19.23±1.91 (14.40-25.00)	1.11±0.08 (0.98-1.42)
Sporocyst	100	15.48±1.42 (12.20-19.00)	10.24±0.96 (8.40-13.00)	1.51±0.13 (1.08-1.83)
Experimental using sporozoites				
Oocyst	100	25.28±2.12 (19.84-29.76)	23.32±2.18 (17.67-27.59)	1.08±0.06 (1.00-1.34)
Sporocyst	100	18.02±1.91 (13.02-21.70)	11.59±1.57 (8.68-19.84)	1.57±0.12 (1.00-1.97)
Experimental using hypnozoites				
Oocyst	100	26.42±1.69 (22.32-30.07)	24.47±1.36 (21.39-27.28)	1.08±0.04 (1.00-1.20)
Sporocyst	100	18.97±1.77 (11.47-22.63)	12.00 (16.43-7.13)	1.59±0.17 (1.13-2.07)

^aNumber of oocysts measured.

The average live weights (ALWs) of the mice in the INFECTED group were consistently lower than those in the CONTROL group, whereas compared with those in the PAIR-FED group, their average live weights followed a similar trend throughout the experiment (Figure 3a). When these parameters were assessed on the days when five mice from each group were euthanized, this observation was less evident. The ALW values of the statistically compared groups are shown in Table 2. The mean weight of the CONTROL group at 15 DAI was significantly greater ($p\leq0.05$) than that of the INFECTED and PAIR-FED groups, whereas at the same time point, the INFECTED and PAIR-FED groups did not significantly differ ($p\geq0.05$). The ALW of the CONTROL group was greater than that of the PAIR-FED group at 35 DAI, with statistically significant differences ($p\leq0.05$) at 15, 16, 17, 18, 26, 27, and 28 DAI. The ALW of the PAIR-FED group did not differ significantly ($p\geq0.05$) from that of the INFECTED group (Table 2).

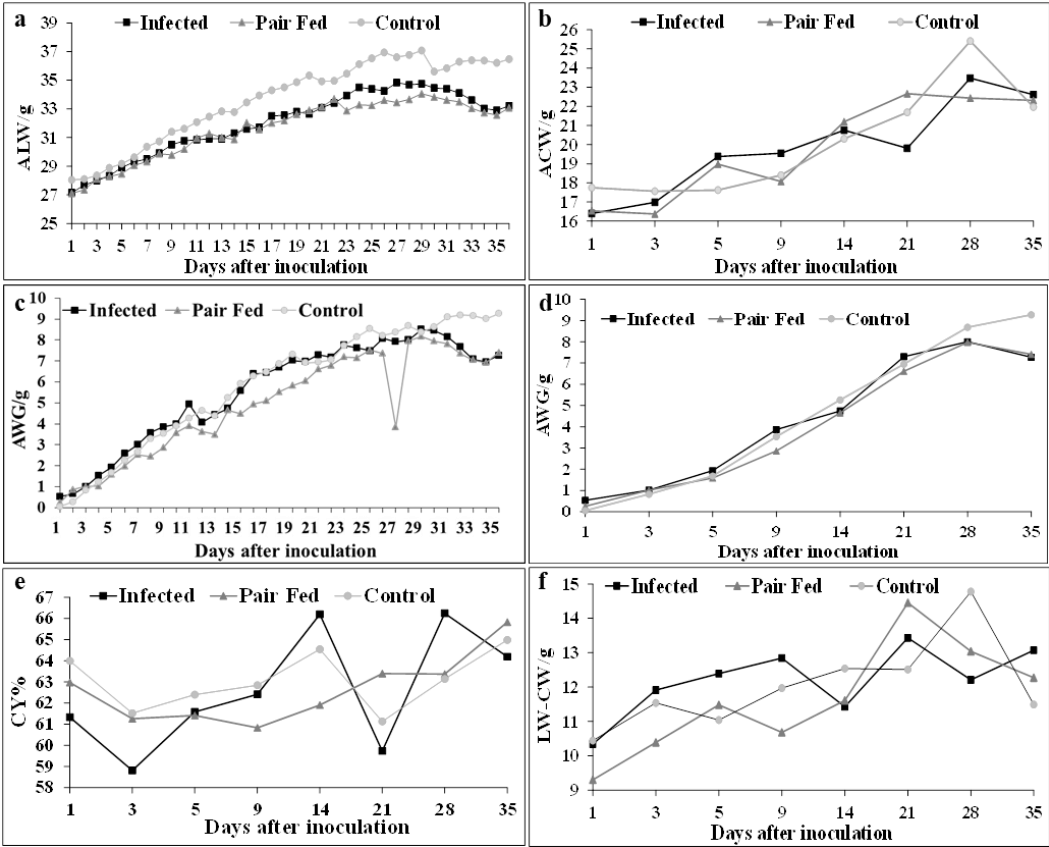


Figure 3. Mean body weight development of mice inoculated with sporulated oocysts of *Cystoisospora ohioensis*-like species (Infected), pair-fed according to the mean daily consumption of their infected group

counterparts (Pair-Fed), and fed ad libitum (Control): (a) Daily average live weight in grams over 35 experimental days (ALW/g); (b) average carcass weight on days when five animals from each group were euthanized (ACW/g); (c) daily average weight gain in grams over 35 experimental days (AWG/g); (d) average live weight gain on days when five animals from each group were euthanized; (e) average percentage carcass yield (CY%) on days when five animals from each group were euthanized; and (f) average live weight minus average carcass weight (Viscera weight) on days when five animals from each group were euthanized.

Table 2. Average live weight in grams (ALW) of mice inoculated with sporulated oocysts of *Cystoisospora ohioensis*-like organisms (INFECTED), fed the average daily intake of their counterparts in the infected group (PAIR-FED), and fed ad libitum (CONTROL).

AI ^a	I ^b n ^b	GROUPS ^c		
		INFECTED	PAIR-FED	CONTROL
0	45	27.22±2.74	27.11±2.22	28.04±2.80
1	45	27.69±2.88	27.28±2.39	28.10±2.60
3	40	28.33±3.18	28.30±2.01	28.88±2.77
5	35	29.30±3.44	29.06±2.30	29.61±2.82
9	30	30.77±2.86	30.19±2.56	31.28±3.22
14	25	31.60±3.04	32.02±3.07	33.44±3.18
15	20	31.70±2.78 (K)	31.57±2.44 (K,Y)	33.94±3.33 (Z)
16	20	32.66±2.70 (KY)	32.02±2.36 (Y)	34.30±3.35 (K,Z)
17	20	32.56±2.44 (KY)	32.18±2.36 (Y)	34.50±3.54 (K,Z)
18	20	32.80±2.25 (KY)	32.62±2.58 (Y)	34.86±3.60 (K,Z)
19	20	32.14±3.20 (K)	32.90±2.66 (K,Y)	35.32±3.64 (Y,Z)
21	20	33.42±2.21	33.75±3.05	34.95±3.93
25	15	34.25±1.83 (K)	33.60±2.45 (K,Y)	36.93±4.50 (Y,Z)
26	15	34.83±2.02 (KY)	33.45±2.44 (Y)	36.61±4.31 (K,Z)
27	15	34.68±2.01 (KY)	33.66±2.59 (Y)	36.76±4.46 (K,Z)
28	15	34.75±2.03 (K,Y)	34.06±2.70 (Y)	37.07±4.34 (K,Z)
35	10	33.22±4.73	33.08±3.42	36.47±4.09

^aDays after inoculation. ^bNumber of samples in each group. ^cLetters, in parentheses, equal in the lines, ALW does not differ according to the Tukey Test (p ≥ 0.05) with a 95% confidence interval.

The average weight gain (AWG) of the mice in the CONTROL group was lower than that of the INFECTED group until the 9th DAI, after which it increased and remained high until the end of the experiment, except at the 16th DAI (Table 3 and Figure 3c). There was no significant difference (p ≥ 0.05) throughout the experiment, except at the 1st DAI, when the AWG of the CONTROL group was significantly lower (p ≤ 0.05) than that of the INFECTED group (Table 3). As observed for ALW, when the AWG parameters were assessed on the days when five mice from each group were euthanized, this trend was less evident (Figure 3d). The PAIR-FED group had AWG values similar to those of the INFECTED group until the 5th DAI, after which its performance became inferior, with statistically significant differences (p ≤ 0.05) on the 8th, 9th, 15th, 16th, 17th, and 27th DAI (Figures 3c and d). Similarly, the performance of the CONTROL group was inferior to that of the PAIR-FED group, with statistically significant differences (p ≤ 0.05) on the 15th, 16th, 17th, 19th, and 27th DAI (Table 3).

Table 3. Average weight gain in grams (AWG) of mice inoculated with sporulated oocysts of *Cystoisospora ohioensis*-like organisms (INFECTED), fed the average daily intake of their counterparts in the infected group (PAIR-FED), and fed ad libitum (CONTROL).

DAI ^a	n ^b	GROUPS ^c					
		INFECTED		PAIR-FED		CONTROL	
1	45	0.53±0.61	(K)	0.25±0.75	(K,Y)	0.05±0.66	(Y,Z)
2	40	0.66±0.76	(KY)	0.87±0.98	(Y)	0.28±0.76	(K,Z)
3	40	1.01±0.98		1.01±0.98		0.82±1.11	
5	35	1.93±1.34		1.58±1.33		1.68±1.12	
8	30	3.58±1.45	(K)	2.45±1.13	(Y,Z)	3.30±1.66	(K,Z)
9	30	3.86±1.47	(K)	2.86±1.28	(Y,Z)	3.53±1.70	(K,Z)
14	25	4.74±2.03		4.65±1.30		5.26±1.54	
15	20	5.59±1.61	(K,Y)	4.48±1.61	(Y)	5.93±1.58	(K,Z)
16	20	6.39±1.53	(K)	4.94±1.69	(Y)	6.29±1.55	(K,Z)
17	20	6.44±1.85	(K)	5.10±1.73	(Y)	6.49±1.56	(K,Z)
19	20	7.03±1.91	(K,Z)	5.82±1.85	(K,Y)	7.32±1.71	(Z)
21	20	7.30±2.05		6.61±1.92		6.95±2.02	
27	15	7.93±1.93	(K)	3.84±1.33	(Y)	8.37±2.32	(K,Z)
28	15	8.00±2.24		7.97±2.22		8.68±2.26	
35	10	7.27±4.71		7.41±3.29		9.27±2.47	

^aDays after inoculation. ^bNumber of samples in each group. ^cLetters, in parentheses, equal in the lines, AWG does not differ according to the Tukey Test (p ≥0.05) with a 95% confidence interval.

Compared with that of the PAIR-FED group, the average carcass weight (ACW) of the mice in the INFECTED group was significantly lower (p≤0.05) on the 21st DAI, whereas on the 28th DAI, the ACW of the PAIR-FED group was significantly lower (p≤0.05) than that of the CONTROL group. At both time points (21st and 28th DAI), the ACW of the INFECTED group was lower than that of the CONTROL group, although the difference was not statistically significant (p≥0.05) (Table 4).

Table 4. Average carcass weights in grams (ACW) relative to live weights of mice inoculated with sporulated oocysts of *Cystoisospora ohioensis*-like organisms (INFECTED), fed the average daily intake of their counterparts in the infected group (PAIR-FED), and fed ad libitum (CONTROL).

DAI ^a	n ^b	GROUPS ^c					
		INFECTED		PAIR-FED		CONTROL	
1	5	16.40±0.65		16.54±1.12		17.75±2.75	
3	5	16.99±1.43		16.38±0.74		17.56±1.65	
5	5	19.38±1.35		18.98±0.36		17.62±1.91	
9	5	19.55±1.05		18.07±1.55		18.40±1.31	
14	5	20.75±3.59		21.19±3.11		20.30±1.65	
21	5	19.81±2.13	(K,Z)	22.66±0.41	(Y)	21.68±0.93	(Y,Z)
28	5	23.47±0.92	(K,Y)	22.43±1.30	(Y)	25.40±1.54	(K,Z)
35	5	22.61±1.00		22.31±2.41		21.96±1.30	

^aDays after inoculation. ^bNumber of samples in each group. ^cLetters, in parentheses, equal in the lines, ACW does not differ according to the Tukey Test ($p \geq 0.05$) with a 95% confidence interval.

Considering the live weight of each individual, the average percentage carcass weight (ACW%) was established. In this case, the ACW% of the CONTROL group was consistently greater than that of the INFECTED group, except on the 14th and 28th DAI. However, these differences were not statistically significant ($p \geq 0.05$). On the 21st DAI, the ACW% of the INFECTED group [$n=5$ ($\bar{X}=59.73 \pm 2.04$) 95% CI] was significantly lower ($p \leq 0.05$) than that of the PAIR-FED group [$n=5$ ($\bar{X}=63.39 \pm 1.76$) 95% CI]. On the same day, the ACW% of the CONTROL group [$n=5$ ($\bar{X}=61.13 \pm 1.93$) 95% CI] was greater than that of the INFECTED group and less than that of the PAIR-FED group, but the difference was not statistically significant ($p \geq 0.05$) (Figure 3e).

To determine whether organ (viscera) weight influences the carcass yield of infected animals, the live weight of each animal was subtracted from its carcass weight (LW-CW). As shown in Figure 3e and comparing it with Figure 3f, an inverse relationship was observed between the average percentage carcass yield (CY%) and viscera weight (LW-CW) in the INFECTED group. This pattern was not observed in the PAIR-FED and CONTROL groups.

No differences were found among animals in the INFECTED, PAIR-FED, and CONTROL groups ($p \geq 0.05$) regarding the average percentage weights of the stomach (SW%) and lungs (LW%) at any of the DAIs when the mice were euthanized throughout the experiment. With respect to the average heart weight (HW%), animals in the CONTROL group [$n=5$ ($\bar{X}=0.85 \pm 0.11$) 95% CI] presented significantly greater HW% ($p \leq 0.05$) than did those in the INFECTED [$n=5$ ($\bar{X}=0.65 \pm 0.11$) 95% CI] and PAIR-FED [$n=5$ ($\bar{X}=0.65 \pm 0.09$) 95% CI] groups, but only on the 9th DAI. On the same day, these latter two groups did not significantly differ ($p \geq 0.05$).

On the 21st DAI, the average percentage weight of the small intestine (AWSI%) relative to the carcass weight (Figure 4a) in the CONTROL group [$n=5$ ($\bar{X}=4.43 \pm 1.40$) 95% CI] was significantly lower ($p \leq 0.05$) than that in the PAIR-FED group [$n=4$ ($\bar{X}=9.39 \pm 1.21$) 95% CI]. During the same period, the AWSI% of the INFECTED group [$n=5$ ($\bar{X}=6.35 \pm 2.8$) 95% CI] was greater than that of both the CONTROL and PAIR-FED groups; however, these differences were not statistically significant ($p \geq 0.05$). When the average percentage weight of the large intestine (AWLI%) relative to the carcass weight was analyzed (Figure 4b), a significant difference was observed on the 1st DAI. The AWLI% of the CONTROL group [$n=5$ ($\bar{X}=10.47 \pm 1.88$) 95% CI] was significantly greater ($p \leq 0.05$) than that of the PAIR-FED group [$n=5$ ($\bar{X}=7.57 \pm 1.35$) 95% CI] but was not significantly different ($p \geq 0.05$) from that of the INFECTED group [$n=5$ ($\bar{X}=9.11 \pm 1.28$) 95% CI].

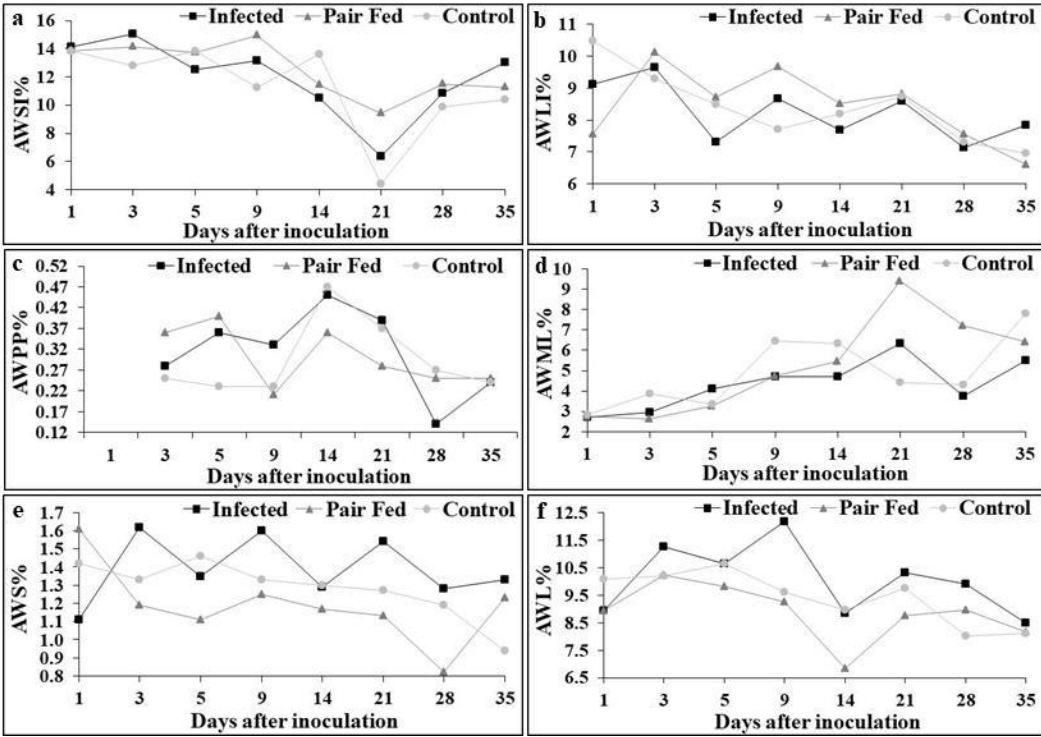


Figure 4. Average percentage weights of the small intestine (AWSI%) (a), large intestine (AWLI%) (b), Peyer's patches (AWPP%) (c), mesenteric lymph nodes (AWML%) (d), spleen (AWS%) (e), and liver (AWL%) (f) relative to the carcasses of five mice inoculated orally with 10^5 sporulated oocysts of *Cystoisospora ohioensis*-like species (Infected), five fed the average daily intake of their counterparts in the infected group (Pair-Fed), and five fed ad libitum (Control) per evaluation day.

Owing to the small size of Peyer's patches in the mice from all three groups, the average percentage weight of Peyer's patches (PPW%) could not be determined on the 1st DAI (Figure 4c). A significantly greater weight ($p \leq 0.05$) of PPW% was observed in the INFECTED group [$n=5$ ($X=0.36 \pm 0.08$) 95% CI] than in the CONTROL group [$n=5$ ($X=0.23 \pm 0.09$) 95% CI] on the 5th DAI (Figure 4c). On the 3rd and 5th DAI, the PPW% of the PAIR-FED group was higher than that of the INFECTED group, although not significantly ($p \geq 0.05$), and subsequently became lower (also not significantly, $p \geq 0.05$) on the 9th, 14th, and 21st DAI. By the 35th DAI, the PPW% values of all three groups were practically identical (Figure 4c).

Changes in the average percentage weight of mesenteric lymph nodes (MLNW%) were more clearly observed from the 9th DAI onward. A significantly greater MLNW% ($p \leq 0.05$) was found between the PAIR-FED [$n=5$ ($X=9.39 \pm 1.21$) 95% CI] and CONTROL [$n=5$ ($X=4.42 \pm 1.40$) 95% CI] groups on the 21st DAI, with values returning to similar levels by the 28th DAI (Figure 4d). On the 28th DAI, mice in the PAIR-FED group presented significantly greater MLNW% ($p \leq 0.05$) values than those in the INFECTED group did [$n=5$ ($X=3.75 \pm 0.35$) 95% CI], whereas no significant difference was observed compared with the CONTROL group [$n=5$ ($X=4.32 \pm 0.82$) 95% CI].

The average percentage spleen weight (SW%) of the animals in the INFECTED group was greater throughout most of the experiment than that of the PAIR-FED and CONTROL groups. Specifically, the SW% of the INFECTED group [$n=5$ ($X=1.33 \pm 0.29$) 95% CI] was significantly greater ($p \leq 0.05$) than that of the CONTROL group [$n=5$ ($X=0.94 \pm 0.09$) 95% CI] but not significantly different ($p \geq 0.05$) from that of the PAIR-FED group [$n=5$ ($X=1.23 \pm 0.21$) 95% CI] at the end of the experiment on the 35th DAI (Figure 4e).

The average percentage liver weight (LW%) of the mice in the INFECTED group increased from the 3rd DAI onward. A significantly greater LW% ($p \leq 0.05$) was observed in the INFECTED group [$n=5$ ($X=12.16 \pm 2.10$) 95% CI] than in the PAIR-FED group [$n=5$ ($X=9.26 \pm 0.77$) 95% CI] on the 9th DAI, whereas no significant difference ($p \geq 0.05$) was found versus the CONTROL group [$n=5$ ($X=9.60 \pm 1.40$)

95% CI] on that day. Compared with those of the other groups, the LW% of the INFECTED group subsequently decreased but remained nonsignificant ($p \geq 0.05$), ultimately reaching similar values among all three groups by the 35th DAI (Figure 4f).

In all the mice from the CONTROL and PAIR-FED groups, no *C. ohioensis*-like hypnozoites were observed in the organs after peptic digestion, nor were the hypnozoites recovered from the stomach, heart, lungs, or liver of the INFECTED group. From this group, a total of 2.4×10^3 hypnozoites were recovered exclusively from the small and large intestines on the 1st DAI, from Peyer's patches between the 1st and 9th DAI, and from mesenteric lymph nodes and the spleen across all 35 evaluated DAIs (Table 5). However, by the 35th DAI, a significant reduction in the absolute number of isolates was observed in mesenteric lymph nodes and the spleen, which was accompanied by a relative decrease in isolation from mesenteric lymph nodes (4.42%) and a relative increase in isolation from the spleen (95.58%). The number of hypnozoites and their percentage relative to the total number of hypnozoites recovered daily from necropsies of various viscera are detailed in Table 5.

The viability of sporocysts in *C. ohioensis* oocysts inoculated into two puppies (designated the SPOROZOITE group) was confirmed when these animals began shedding a total of 9×10^5 oocysts over 24 days starting from the 5th DAI (Figure 1b). Similarly, the viability of hypnozoites present in monozyotic tissue cysts from the viscera of mice infected with 1×10^5 sporulated oocysts was demonstrated when the other two puppies (designated the HYPNOZOITE group) shed a total of 16.2×10^5 oocysts over 27 days starting from the 4th DAI (Figure 1b). Neither of the two puppies inoculated orally with the same volume of inoculum (0.5 mL of PBS) containing organs from the CONTROL or PAIR-FED group mice shed oocysts in their feces. All six puppies maintained a healthy appearance without signs of infection or notable gastrointestinal symptoms. The mean dimensions and standard deviations of MD, md and MI, along with the maximum and minimum measurements of oocysts and sporocysts recovered from the HYPNOZOITE and SPOROZOITE groups, are shown in Table 1. The MD measurements of oocysts from the HYPNOZOITE group were significantly greater ($P \leq 0.05$) than those from the SPOROZOITE group, whereas the md and MI measurements were not significantly different ($P \geq 0.05$) between the groups. The daily oocyst shedding patterns were similar between the HYPNOZOITE and SPOROZOITE groups until the 21st DAI, when the HYPNOZOITE group presented a renewed increase in oocyst excretion (Figure 1b).

Table 5. Hypnozoites quantified by recovery from the viscera of five mice inoculated with sporulated oocysts of *Cystoisospora ohioensis*-like organisms.

DAI ^a	HYPOZOITES/VISCERA										TOTAL	
	Small intestine		Large intestine		Peyer's patch		Mesenteric lymph node		Spleen			
	n ^b	%	n	%	n	%	n	%	n	%	n	%
1	102	17.62	147	25.39	135	23.31	86	14.85	109	18.83	579	100
3	-	-	-	-	50	7.17	506	72.60	141	20.23	697	100
5	-	-	-	-	28	25.00	44	39.29	40	35.71	112	100
9	-	-	-	-	9	4.89	60	32.61	115	62.50	184	100
14	-	-	-	-	-	-	38	18.45	168	81.55	206	100
21	-	-	-	-	-	-	60	23.90	191	76.10	251	100
28	-	-	-	-	-	-	35	12.96	235	87.04	270	100
35	-	-	-	-	-	-	5	4.42	108	95.58	113	100
GRAND TOTAL	102	4.23	147	6.09	222	9.20	834	34.58	1.107	45.90	2.412	100

^aDays after inoculation. ^bNumber of hypnozoites per viscera.

4. Discussion

Coccidiosis is a common condition in dogs, and the most frequent clinical aspects are depression, weakness, loss of appetite, diarrhea, and dehydration [12,48–54]. Clinical coccidiosis was confirmed

in a 10-week-old dog, which exhibited weight loss and yellowish and diarrheic feces, with the condition progressing to death despite therapeutic assistance with sulfaguanidine. Severe enteritis is associated with the asexual and sexual stages of organisms resembling *C. ohioensis*-like organisms [55]. There are few reports of *C. canis* causing coccidiosis in dogs; however, Mitchell et al. [56], upon experimentally infecting dogs with 1×10^5 oocysts, reported weight loss, diarrhea, vomiting, and lethargy. Since other enteric etiological agents are responsible for similar clinical signs, a positive response to anticoccidial therapy has helped determine possible diagnoses [53]. However, a favorable response to anticoccidial therapy as support for the diagnosis of clinical coccidiosis is considered misleading by Kirkpatrick and Dubey [57], as most anticoccidial drugs also possess antibacterial properties. He et al. [58] did not observe any signs or symptoms of coccidiosis in 20 dogs that had sporulated oocysts of *C. ohioensis*-like, where at least one of these dogs was molecularly confirmed to have *C. ohioensis*. More recently, Barrera et al. [59], upon examining 524 fecal samples from dogs in a kennel over one year, diagnosed 46 (8.8%) animals as positive for *Cystoisospora* spp. Of these, the positivity rate among mothers was 10.6% (32/300), and that among litters was 6.2% (14/224). The *Cystoisospora* species identified were *C. canis* in 91.3% (42/46) of the samples and *C. ohioensis*-like species in 8.7% (4/46). Throughout the research period, none of the animals exhibited signs of coccidiosis. Similarly, in the present study, none of the dogs—whether naturally infected, infected with 1×10^5 sporulated oocysts, or fed viscera containing hypnozoites from mice previously infected with 1×10^5 sporulated oocysts—exhibited any signs or symptoms related to coccidiosis. These results may be related to the distinction of endogenous developmental stages, with *C. ohioensis*, according to Dubey [60], being confined to the superficial intestinal epithelium, which differs from *C. rivolta* and *C. burrowsi*, whose endogenous stages occur predominantly in the lamina propria. Thus, we cannot infer that the naturally infected dogs and oocyst donors in this study were or had been infected by *C. ohioensis* but rather by the group of medium-sized coccidia referred to as the *C. ohioensis*-like complex.

Oocysts were shed in significant numbers by all six naturally infected puppies, as well as by the two puppies fed hypnozoites contained in the viscera of previously infected mice and by the two puppies orally infected with sporozoites contained in sporulated oocysts (Figures 1a and 1b). The number of oocysts shed daily by the dogs in the groups inoculated with hypnozoites and sporozoites was similar until the 21st DAI (days post infection), when the HYPNOZOITES group presented a new increase in the number of oocysts shed (Figure 1b). The animals in this group reached peak daily shedding on the 25th DAI and continued shedding oocysts until the 27th DAI, differing from the animals in the SPOROZOITE group, which peaked on the 5th DAI and shed oocysts until the 24th DAI (Figure 1b).

The prepatent period of *C. ohioensis* can range from 13 to 23 days [36,61], and the prepatent period observed by Rocha & Lopes [62] in dogs inoculated with sporulated oocysts was 3 to 7 days. In induced infections, the observation of gametocytes in the intestinal cells of dogs at 96 and 114 hours postinoculation with hypnozoites and sporozoites, respectively, suggests a biological distinction between the two parasitic forms [63]. Dubey and Lindsay [8], in their review of canine coccidiosis, reported that the asexual and sexual stages in cycles induced by sporozoites or hypnozoites are structurally similar in size and location, but the hypnozoite-driven cycle develops 24 hours faster. This was evident in our research results, where dogs inoculated with hypnozoites had a shorter prepatent period, shed a greater number of oocysts, and exhibited a longer patent period, reinforcing the hypothesis that the two parasitic stages are biologically distinct, as suggested by Dubey [63] and Dubey & Lindsay [8]. The oocyst shedding pattern in naturally infected dogs was similar to that in dogs fed viscera from previously infected mice (hypnozoites), suggesting that the six naturally infected dogs had ingested hypnozoites through contaminated food. In 1995, Dubey reported that dogs infected with 1×10^5 oocysts of *C. canis* or fed tissues from mice previously infected with 1×10^5 oocysts produced similar numbers of oocysts and had comparable prepatent periods [64]. However, in our experiment, dogs inoculated with 1×10^5 sporulated oocysts had a longer prepatent period than those fed viscera from mice previously inoculated with 1×10^5 sporulated oocysts (Figure 1b).

The subspherical appearance of the oocysts and ellipsoid shape of the sporocysts shed by the six naturally infected dogs, as well as the measurements of the major and minor diameters of the oocysts and sporocysts, were compatible with those observed by Rocha & Lopes [62] and Dubey [65] for *C. rivolta* and with those characterized by Lindsay et al. [7] as intermediate-sized oocysts from dogs with dimensions ranging from 17 to 23 μm , grouped as *C. ohioensis*-like (Figure 1a). In the present study, the measurements of MD, md and MI of oocysts and sporocysts from the six naturally infected dogs were smaller (Table 1) than those of oocysts and sporocysts shed by the two puppies inoculated with sporulated oocysts and the two puppies fed mouse viscera containing hypnozoites. These differences in measurements may be related to varying proportions of medium-sized oocysts being shed, corresponding to the species *C. ohioensis*, *C. burrowsi* and *C. rivolta*, which cannot be distinguished morphologically [7].

The lower ALW of the animals in the INFECTED group throughout the experiment, which became statistically significant from the 15th DAI compared with that of the CONTROL group (Table 2), and the AWG of the PAIR FED group, which was consistently lower than that of the INFECTED group (Table 3), support the hypothesis that the reduced performance of intermediate hosts in terms of daily live weight and weight gain is related to the development of infective forms for the subsequent establishment of extraintestinal hypnozoites, where an increase in visceral weight (Figure 3f) when parasitized masks the wasting effect of *C. ohioensis*-like species. These observations were also detected in mice inoculated with sporulated oocysts of *C. rivolta* (= *C. ohioensis*-like) by Brösike et al. [66] and with *C. felis* by Loss and Lopes [67]; Freire and Lopes [68] and Costa and Lopes [69]. When Costa and Lopes [69] inoculated rabbits with sporulated oocysts of *C. felis*, they reported that the animals lost weight from the first week after infection until the end of the experiment on the 29th DAI. The differences in ALW and AWG between the INFECTED and CONTROL groups are likely related to the anorexia that follows infection, as described by Loss and Lopes [67].

The consistently lower ALW of the animals in the INFECTED group throughout the experiment, which became statistically significant from the 15th DAI compared with that of the CONTROL group (Table 2), along with the AWG of the PAIR-FED group being consistently lower than that of the INFECTED group (Table 3), supports the hypothesis that the reduced performance of intermediate hosts in terms of daily body weight and weight gain is related to the development of infection stages, leading to the establishment of extraintestinal hypnozoites. In this scenario, the increased visceral weight (Figure 3f) when parasitized masks the cachectic effect of *C. ohioensis*-like infection. Similar observations were reported in mice inoculated with sporulated oocysts of *C. rivolta* (= *C. ohioensis*-like) by Brösike et al. [66] and with *C. felis* by Loss and Lopes [67], Freire and Lopes [68], and Costa and Lopes [69]. When Costa and Lopes [69] inoculated rabbits with sporulated oocysts of *C. felis*, they observed weight loss starting from the first week postinfection until the end of the experiment on the 29th DAI. The differences in ALW and AWG between the INFECTED and CONTROL groups are likely related to postinfection anorexia, as described by Loss and Lopes [67].

Studies with cattle [70,71]; buffalo, sheep, and swine [17,21,23]; rabbits [20]; and broiler chickens [22] have indicated that production animals can harbor varying numbers of *Cystoisospora* genus hypnozoites in their viscera. However, whether parasitic action in these organs influences weight gain in these animals or causes significant lesions remains unknown. Nevertheless, recurrent clinical disease is common in both immunocompetent and immunosuppressed humans infected with *C. belli*, which is believed to be due to reactivation of the hypnozoites present in tissues and their migration to the human intestinal tract [24,31]. Immunosuppressed patients frequently experience clinical disease relapse after discontinuation of *C. belli* treatment [69], although Frenkel et al. [71] failed to infect suckling calves and chicks with 1×10^6 and 3×10^4 sporulated oocysts of *C. belli*, respectively. Ultimately, the interference of this parasitism on weight development in humans and animals remains unknown, whereas economic production of animal protein requires increasingly greater financial investments to meet the demand for meat, viscera, or both. Our research suggests that animals may have impaired zootechnical performance, as occurred with mice in the INFECTED group during the establishment of hypnozoites in target organs or due to anorexia induced during

this process. This hypothesis is reinforced by the fact that mice in the PAIR-FED group of our experiment showed lower ALW and AWG performance than the INFECTED group, resulting from feed control that increased stress levels and the lack of nutrients necessary for the development of healthy animals.

Biological assays in cats have indicated that infectious stages of *C. felis* and *C. rivolta* can develop in gerbils (*Meriones unguiculatus*), guinea pigs (*Cavia porcellus*), rabbits (*Oryctolagus cuniculus*), and chickens (*Gallus gallus domesticus*) fed sporulated oocysts [74]. In addition to the results of this study, it can be inferred that infection of mice with *C. ohioensis*-like and potentially other intermediate hosts negatively affects carcass weight. The increased organ weight in the parasitized animals (Figure 3f) contributed to a lower carcass yield (Tables 3 and 4), since parasitism also reduced the ALW (Figures 3a, b and Table 2) and AWG (Figures 3c, d and Table 3) of the infected mice.

Freire and Lopes [68] reported no differences in the mean weight percentages of the heart, lungs, kidneys, and brain in mice inoculated with sporulated oocysts of *C. felis*. Dubey [11] previously failed to demonstrate hypnozoites or extraintestinal stages of *C. ohioensis* in histopathological sections of the heart, lung, and brain, which led Freire and Lopes [66] to suggest that these organs, including the kidneys, are rarely parasitized by *Cystoisospora* species. Brösike [75] studied the frequency of *C. rivolta* (= *C. ohioensis*-like) hypnozoites in Swiss albino mice, and although this parasite is widely distributed throughout the host organism, it does not develop in the brain. In our research, no differences in ASW% (average stomach weight percentage) or AWH% (average weight heart percentage) were detected between the groups throughout the experiment. In fact, the AWSI% and AWLI% of the mice in the INFECTED group were greater, although not significantly so, over 35 days, likely because of the presence of transient parasites in these organs, as shown in Table 5. Considering the viscera where *Cystoisospora* spp. has been previously reported [66–69,75,76] and the ASW%, AWH%, AWPP% (percentage of the average weight of the Peyer's patch), AWSI%, and AWLI% observed in this experiment, these organs are not frequently parasitized by *C. ohioensis*-like organisms, as previously reported with other *Cystoisospora* species by these same researchers. All the aforementioned authors reported alterations in the AWPP%, AWML% (percentage of the average weight of the mesenteric lymph node), AWS% (percentage of the average weight of the spleen), and AWL% (percentage of the average weight of the liver) of animals inoculated with *Cystoisospora* spp., which is consistent with our observations for *C. ohioensis*-like organisms. Dubey [76] demonstrated that sporozoites released after the excystation of *C. rivolta* sporulated oocysts most frequently invade mouse mesenteric lymph nodes, which remain infected for at least 23 months, potentially explaining the increased AWML% returning to proportionality in our study (Figure 4d), as also verified by Freire and Lopes [68] between the 4th and 18th DAI, with this finding being attributed to the distribution of parasite hypnozoite forms to host organs. These researchers reported increased AWL% in infected mice from the 3rd to 18th DAI, whereas in our experiment, the AWL% increase occurred from the 3rd to 9th DAI (Figure 4f), followed by a decrease, with no differences among the INFECTED, PAIR-FED and CONTROL groups until the 35th DAI. These cyclic alterations, also observed in AWPP% and AWML%, may be due to spontaneous flow of parasite hypnozoite forms during their biological cycle development in the paratenic host, which is consistent with Dubey [76], who demonstrated that hypnozoites increased in size during the first 31 days and could be found in the spleen, liver, and skeletal muscle of mice infected with *C. rivolta* (= *C. ohioensis*-like). It has also been shown that the hypnozoites of *C. felis*, *C. rivolta* and *C. ohioensis* increase in size over time but do not divide [10,18]. Our results revealed that during parasite passage through organs, their weight percentages increased (Figures 4a,f), possibly due to elimination of some forms while others became encysted. Since parasite multiplication does not occur in the intermediate host, the organ weight percentages returned to normal during the chronic infection process, as observed in this experiment, with AWPP% (Figure 4c), AWML% (Figure 4d) and AWL% (Figure 4f) values. However, the spleen did not follow this pattern, with AWS% (Figure 4e) increasing throughout the entire process, including the chronic phase, suggesting that this organ best harbors and maintains *C. ohioensis* parasitic forms for the longest duration.

Parasite dissemination in rodent organs occurs similarly to that in other coccidia through phagocytes [10,77–79]. Freire and Lopes [68] suggested the existence of both hematogenous and lymphatic routes for extraintestinal dissemination of *Cystoisospora* species in intermediate hosts. Liver and spleen enlargement in the initial days postinoculation results from rapid parasite spread via the bloodstream, whereas slower dissemination likely occurs through the lymphatic system, sequentially increasing lymphoid organ size. According to Lindsay et al. [80], further investigations are needed to elucidate the complex life cycle and epidemiology of *Cystoisospora* spp., as developmental cycles were originally described in immunocompetent hosts, and the impact of immunosuppression on definitive host development remains unknown. Our research revealed temporary production losses in immunocompetent intermediate hosts, which are likely exacerbated in immunocompromised animals—a common scenario in meat and dairy production systems due to the confinement stress required for increased productivity.

Freire and Lopes [68] observed *C. felis* hypnozoites in the kidneys, lungs, and heart, attributing the presence of the parasite in these organs to accidental distribution rather than a normal parasitic access route. The absence of hypnozoites in the stomach, heart, and lungs in our study suggests that these organs are not preferential for *C. ohioensis*-like organisms in mice, a hypothesis supported by visceral weight evaluation findings. No hypnozoites were observed in the livers of INFECTED group mice in our research. Considering that the liver is a known site for hypnozoite observation in intermediate hosts infected with *C. rivolta* [66,75] and *C. felis* [68,69], we cannot conclusively state that the liver was free from *C. ohioensis*-like parasitism in our study, as a significant cellular mass remained after the peptic digestion of liver tissue, requiring substantial sample dilution to improve hypnozoite visualization. Furthermore, hypnozoite recovery in the spleen throughout the experiment, particularly at 1st DAI (Table 5), supports the hematogenous dissemination hypothesis [68] previously discussed in visceral weight analysis. Therefore, a more comprehensive evaluation of the presence of *C. ohioensis*-like hypnozoites is needed to definitively exclude liver parasitism in experimental animals.

In the spleen, a progressive increase in both the absolute and relative numbers of recovered hypnozoites was observed, indicating that this organ is the primary target of *C. ohioensis*-like organisms in mice. Brösigke [75] noted a strong predilection of *C. rivolta* for mesenteric lymph nodes at the 2nd, 4th, 84th, and 168th DAI, whereas the liver showed a preference only at the 3rd and 7th DAI. Freire and Lopes [68] reported that *C. felis* prefers the spleen and liver, with considerable hypnozoite recovery in Peyer's patches and mesenteric lymph nodes, suggesting that *C. rivolta* is more lymphotropic than *C. felis* is. Velásquez et al. [27] reported the clinical manifestations, humoral responses, and histological findings of different stages during *C. belli* infection in an AIDS patient with liver disease. These findings led researchers to hypothesize that sporozoites and/or monozyotic tissue cysts invade duodenal mucosal epithelial cells, penetrating the basement membrane and lamina propria. The authors further suggest that monozyotic tissue cysts likely migrate to the liver via portal blood circulation, although lymphatic vessel migration cannot be ruled out [27]. The dynamics of *C. ohioensis*-like organisms in our study indicate a faster hematogenous route to the spleen between the 1st and 3rd DAI, as evidenced by increased hypnozoite recovery at these timepoints (Table 5), and a slower lymphatic migration route, as shown by increasing hypnozoite counts from the 9th DAI onward (Table 5). These results suggest that the *C. ohioensis*-like distribution is more lymphotropic, which aligns with Freire and Lopes' [66] observations for *C. rivolta*. Although the spleen was the target organ for *C. ohioensis*-like organisms in our study, decreased parasite recovery at the 35th DAI (Table 5) may reflect either parasite elimination during its biological cycle or survival bias of acid-pepsin digestion-resistant stages [78]. These findings support the immunological regulation hypothesis, where *C. rivolta*-like species are more easily controlled than *C. felis* are [70,81].

5. Conclusions

Studies analyzing the deleterious effects of *Cystoisospora* spp. are scarce, and no research has addressed the pathogenic, biological, and nutrient-depleting aspects related to *C. ohioensis*-like

organisms in intermediate hosts. However, studies on other coccidia, such as those of the genera *Toxoplasma*, *Eimeria*, *Sarcocystis*, and, more recently, on *Neospora* and *Cystoisospora belli* in humans, have been conducted, highlighting the importance of these coccidia in animal production and One Health. The role of coccidia as opportunistic agents is well known, and this aspect has gained significance with the emergence of immunosuppressive diseases such as AIDS and the stresses of modern life. The medications used in chemotherapy and organ transplant rejection control, along with the indiscriminate use of corticosteroids in veterinary medicine, render both humans and animals more vulnerable to pathogens once they are considered nonpathogenic. In livestock, the stress of confinement systems aimed at achieving high productivity in certain farming operations is a key factor in opportunistic infections. In the intermediate host, *C. ohioensis*-like species invade extraintestinal organs, being transported via the lymphatic system and—although suggested but not confirmed in this study—potentially through the bloodstream as well. The parasitized organs increase in size, masking the body weight and weight gain of the animals and ultimately impairing carcass yield. These findings demonstrate the nutrient-depleting and nutrient-wasting effects of *C. ohioensis*-like parasitism in intermediate hosts. Most transport hosts are optional for parasites and are considered "carriers" without true parasitism—meaning a harmonious parasite–host relationship. However, on the basis of the results of this study, nondefinitive hosts of *C. ohioensis*-like species should be classified as intermediate hosts rather than transport or paratenic hosts, as the latter do not support parasite development or exhibit any host effects.

Author Contributions: “Conceptualization, F.C.R.O., S.S.M.G. and C.W.G.L.; methodology, F.C.R.O., S.S.M.G. and C.W.G.L.; software, F.C.R.O.; validation, F.C.R.O., S.S.M.G. and C.W.G.L.; formal analysis, S.S.M.G. and C.W.G.L.; investigation, F.C.R.O.; resources, C.W.G.L.; data curation, F.C.R.O., S.S.M.G.; writing—original draft preparation, F.C.R.O.; writing—review and editing, S.S.M.G. and C.W.G.L.; visualization, S.S.M.G.; supervision, C.W.G.L.; project administration, F.C.R.O. and C.W.G.L.. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted in accordance with the subsequently established Law No. 11,794/2008 (which revoked Law No. 6,638 of May 8, 1979; [44]) and CFMV Resolution No. 714/2002 [45].

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Conflicts of Interest: The authors declare no conflicts of interest.

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