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Eimeria Legionensis and *Eimeria kofoidi* (Apicomplexa: Eimeriidae) Infection and Associated Lesions in Naturally Infected Red-Legged Partridges (*Alectoris rufa*)

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Abstract: With the aim to identify the *Eimeria* species responsible for coccidiosis in 50 deceased red-legged partridges (*Alectoris rufa*), individual faecal samples were collected, dissolved in 2.5% $K_2Cr_2O_7$ solution and maintained at room temperature to allow sporulation of the oocysts. Morphology and dimensions of sporulated oocysts were microscopically evaluated. To assess *Eimeria* intestinal localisation, faecal samples and scrapings taken from the different intestinal segments of each deceased animal were examined by fresh smears and flotation test, while the intestines were examined for gross lesions, then fixed in 10% formalin and processed for histopathological analysis. From scrapings and morphological analysis, *Eimeria kofoidi* and *Eimeria legionensis* were identified in the small intestine and in the caecum and colon, respectively. Histopathological analysis confirmed the presence of two distinct *Eimeria* species. In particular, *E. kofoidi* macrogamonts were found in epithelial cells of jejunum and ileum, between the basal lamina and the nucleus of the infected intestinal cells. This latter was flattened and displaced above. *E. legionensis* macrogamonts were instead found localised between the nucleus and the luminal surface of the infected caeca and colonic cells and these macrogamonts were larger than those of *E. kofoidi*. Chronic enteritis and severe displacement of the deep crypts of the small intestine, large areas of caeca and colonic epithelial necrosis associated to thickened wall and mononuclear cells infiltration diffused in a transmural manner, were the main histopathological lesions.

Keywords: *Eimeria Kofoidi*, *Eimeria Legionensis*, Intestinal Lesions, Intestinal Localisation, *Alectoris Rufa*

Introduction

Among Galliformes, the red-legged partridge (*Alectoris rufa*) is an important phasianid game bird species (Birkan and Jacob, 1988) native to the Iberian Peninsula, but currently presents also in Italy (Millan, 2009; Naciri *et al*., 2011). Among birds of the genus *Alectoris*, coccidiosis is a disease caused by intracellular intestinal protozoans of the genus *Eimeria* (Apicomplexa: Eimeriidae). Coccidiosis is prevalent in game farms and often responsible for diarrhoea, weight loss, poor feed conversion and for mortality of infected birds (Pellérdy, 1974; Reck and McQuistion, 1994). Clinical disease or even epidemic-like outbreaks with considerable mortality are frequently observed in game farms (Bolognesi *et al*., 2006; Naciri *et al*., 2011), especially when birds are reared on the floor and kept in confined pens that favour the spread of coccidian infections. Although coccidiosis of the red-legged partridge is known from a long time (Pellérdy, 1974; Levine, 1988; Duszynski *et al*., 2000; Bolognesi *et al*., 2006; Naciri *et al*., 2011), few data are available on histopathological lesions caused by *Eimeria* species infecting *A. rufa* and on the histological localisation of these species.

This study was aimed to assess *Eimeria* species and associated lesions in naturally infected and deceased A. *rufa*, reared for their releasing in the natural habitat.

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Materials and Methods

Animals

Fifty deceased red-legged partridges (*A. rufa*) of about 30-40 days in age and from a farm located in Tuscany (central Italy), were examined. In this farm, birds are reared directly on the floor through their life and coccidiosis is frequently observed.

Examined birds had died after a coccidiosis outbreak with about 10% mortality.

Parasitological and Gross Examination

From each examined bird, rectal faecal samples were collected. An aliquot of each faecal sample was microscopically observed fresh and after flotation test, by using a low density solution (specific gravity 1.2). To allow sporulation of *Eimeria* oocysts, the remaining aliquots were dissolved in 2.5% $K_2Cr_2O_7$ solution at 22°C ± 1°C. After sporulation, oocysts, sporocysts and all other structures were microscopically evaluated at 400× and 1000× and measured by using a 10× eyes pie micrometer.

In order to evaluate the presence of gross lesions due to coccidiosis, at necropsy the intestines were removed and opened from the duodenum to the rectum. In addition, faecal contents and scrapings of intestinal mucosa taken from each intestinal segment, i.e. proximal and distal duodenal, jejunal and ileal tracts, caecum and recto-colic tracts, were microscopically evaluated as fresh samples and after flotation test, by using a low density solution (specific gravity 1.2). A faecal aliquot taken from each intestinal segment was also processed for sporulation and identification of the isolated oocysts, as described above.

Histopathological Examination

From all necropsied birds, the different intestinal segments were fixed in 10% buffered formalin and then paraffin embedded. Three micrometers thick sections were placed onto pre-treated slides (Bio-Optica, Milan, Italy) to promote adhesion, and dried overnight at 37°C. After being de-waxed, sections were stained with hematoxylin-eosin (HE) for histopathological examination. All the histological samples were microscopically examined at 100x and 400X magnification to identify the intestinal segment/s infected by *Eimeria* species and to score coccidian infection as the mean number of parasites counted in up to 10 fields per section (100 cells counted per field) at 400X magnification (Perrucci *et al*., 2006).

Histopathological intestinal lesions were scored according to the Sydney System classification (Genta and Dixon, 1995). To this aim, the number of inflammatory cells found in the different intestinal compartments, i.e., villus, basal crypt area, villus-crypt junction of the small intestine and apical crypt area and basal crypt area of the large intestine, was microscopically quantified by using a 40× objective, a 10× eyepiece and a square eyepiece reticule (10×10 squares, with a total area of 62,500 μm^2). Then, arithmetic means were calculated for ten 62,500 μm^2 sites of each intestinal region.

Histological criteria for normal gastrointestinal mucosa and wall included detection of none or only a few mononuclear cells interspersed throughout the corion, absence of lymphoid aggregates and none or only a few scattered neutrophils across the intestinal epithelium, per High-Power Field (HPF) (Rossi *et al*., 2015; Fronte *et al*., 2013).

Neutrophils were classified as absent (score 0) when there was none or only single sporadic cells, mild (score 1) for 5 to 10 cells, moderate (score 2) for 20 to 40 cells, marked (score 3) for 50 to 100 cells and severe (score 4) for 100 to 200 cells or more, per HPF. The number of mononuclear cells was considered to be normal (score 0) when none or only a few cells were seen among intestinal glands, mild (score 1) for 50 to 100 cells, moderate (score 2) for 100 to 200 cells, marked (score 3) for 200 to 600 cells and severe (score 4) for 600 cells or more), per HPF.

The number of lymphocyte aggregates was also counted and the status of "activation" of follicles of lymphocyte aggregates was scored by measuring the mean of the areas of 10 randomly selected follicles in each infected partridge.

Results

Parasitological and Gross Examination

After sporulation, all examined animals were found positive for *Eimeria* coccidian oocysts of two different morphology. From microscopically evaluation of oocysts found in faecal samples and in faecal contents and scrapings of each intestinal segment, the first type of oocysts (n = 1000) were found in the jejunum and ileum (Table 1). Gross lesions found in these intestinal tracts included the presence of mucus and erosions. Thickening of the wall of the posterior half of the small intestine was also observed.

Oocysts found in these intestinal segments are oval in shape and measure 17.60 μm × 13.26 μm in size (14.04-19.50 μm × 11.56-14.82 μm), with a length/width ratio of 1.33 (1.00-1.91). No residuum was present, but one or more polar granules were present in mature oocysts. The almond-shaped sporocysts measure 8.46 μm × 6.00 μm (6.24-10.92 μm × 4.68-7.80 μm) with a length/width ratio of 1.41 (1.00-2.00) and show a granular residuum and a small Stieda body (Fig. 1A). Because of these features and of intestinal localisation, these oocysts were identified with *Eimeria kofoidi* (Table 2).

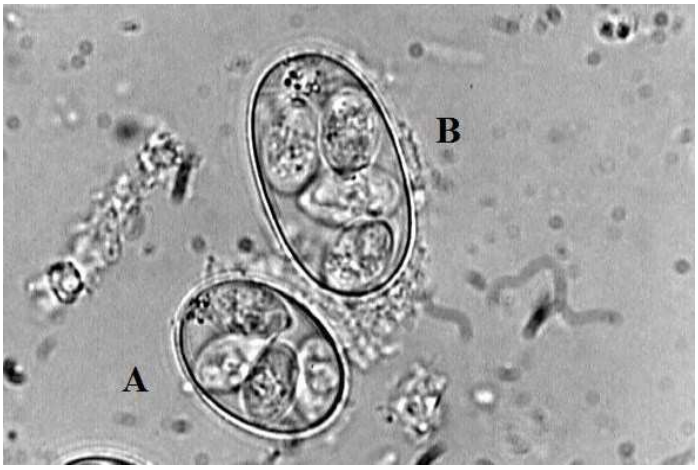

Fig. 1: (A) *Eimeria kofoidi* sporulated oocyst (B) *Eimeria legionensis* sporulated oocyst

Table 1: Mean score (± standard deviation) of infection intensity and of histopathological lesions found in the different intestinal segments of 50 deceased red-legged partridges (*Alectoris rufa*) infected by *Eimeria kofoidi* and *Eimeria legionensis*

Intestinal segment	*Eimeria* species	Infection Score*	Lesion Score**
Duodenum	-	0 (± 0)	0 (±0)
Jejunum	*E. kofoidi*	1.4 (± 0.50)	1.2 (±0.75)
Ileum	*E. kofoidi*	3 (± 0)	3.4 (±0.50)
Caecum	*E. legionensis*	2.8 (± 0.40)	3.6 (±0.50)
Colon	*E. legionensis*	1.8 (± 0.48)	0.8 (±0.40)

*Score: **0**: no infected enterocytes; **1**: <20% of infected enterocytes; **2**: 20% - 40% of infected enterocytes; **3**: >40% infected enterocytes** Mean of the scores found at histopathological examination for each type of inflammatory cells (lymphocytes and lymphocyte aggregates, macrophages and neutrophils) in ten areas of 62,500 μm^2 of each intestinal segment

Table 2: Comparison of *Eimeria kofoidi* and *Eimeria legionensis* oocyst morphology and dimensions (in μm) and their intestinal localisation observed in 50 deceased red-legged partridges (*Alectoris rufa*) examined in the present study, with those reported in previous studies from the same bird host

Species	Mature Oocysts Morphology and dimensions (range)	Sporocysts Morphology and mean mean dimensions (range)	Localisation	References
Eimeria kofoidi	Oval; 1 or 2 PG 17.60×13.26 (14.04-19.50×10.56-14.82)	Ovoid almond; R; SB 8.46×6.00 (6.24-10.92×4.68-7.80)	Jejunum and Ileum	Present Study
	Oval; 1PG 19×13.3 (13.6 -20.9×7.6-15.2)	Elongated; R; SB 9.1× 5.2 (7.6-11.4×4.7-7.6)	Terminal Ileum and Caecum	Lizcano Herrera and Romero Rodriguez (1972)
	Rounded 17.4 X 14.91 (16-19×14-16)	Oval 8.5× 5.5	_*	Bolognesi *et al.* (2006)
	Spherical/Broadly Ovoid; 1, 2 or more PG 19.3-16.3 (14.0-21.4×12.0-19.5)	Ovoid almond; SB 9.4×5.3	Small intestine, mainly in the Duodenal loop and Jejunum	Naciri *et al.* (2011)
Eimeria legionensis	Elliptic; M 22.58×15.12 (15.60-27.30×12.48-17.94)	Almond-shaped; R; SB 9.95×6.31 (7.02-10.92×4.68-7.02)	Caecum and Colon	Present Study
	Elliptic, M 21.3×14.6 (18-24×12-16)	Almond-shaped; R; SB 9.5-10.6	Caecum	Cordero Del Campillo and Hernàndez (1966)
	_ 18.5×14.2	_ _	_	Hernandez Rodriguez *et al.* (1974)
	Elliptic, M 22.0×15.3 (19-24×13-16)	Almond-shaped; SB 9.73×5.56	_*	Bolognesi *et al.* (2006)
	Elliptic, M 22.6×14.9 (18.6 – 26×13-16.7)	Almond-shaped; R; SB 9.8×6.2	Caecum	Naciri *et al.* (2011)

*: Faecal cultures carried out on the whole intestinal content without distinguishing the different tracts, but macroscopic lesions were mostly found in the duodenum; PG: Polar Granules; R: granular Residuum; SB: Stieda Body; M: Micropyle

The second species was found mainly in the caecum, but also in the colon (Table 1). Gross lesions in the large intestines included oedema, pinpoint hemorrhages and thickening, mainly of the caecal wall. These oocysts (n = 1000) are elliptic and of 22.58 µm × 15.12 µm (15.60-27.30 µm × 12.48-17.94 µm) in size, with a length/width ratio of 1.49 (1.11-1.94). No residuum appeared after sporulation, but a micropyle is visible in the oocyst wall. The sporocysts measure 9.95 µm × 6.31 µm (7.02-10.92 µm × 4.68-7.02 µm) with a length/width ratio of 1.58 (1.29-2.00). They contain a granular residuum and show an evident Stieda body at the pointed end (Fig. 1B). Based on their morphological and metrical features and intestinal localisation, these oocysts were identified with *Eimeria legionensis* (Table 2).

Histopathological Examination

Histopathological analysis confirmed that all examined birds were infected by two different *Eimeria* species. At histopathological analysis, jejunum, ileum, caecum and colon were the intestinal sites of coccidian localisation and lesions, although with various degrees of infection and lesion score (Table 1 and 3). In particular, lesions found in the small intestine were represented by chronic enteritis and mononuclear cells infiltration diffused in a transmural manner in the jejunal and ileal walls. The ileum was the site in which the higher degrees of infection and lesion scores were observed (Table 1 and 3). In some cases, a severe displacement of the deep crypts (Fig. 2) associated with the presence of a large number of different *Eimeria* life stages, indicating an active coccidian replication was evidenced. Among the different coccidian stages, macrogamonts observed in the crypts of the ileum and in the jejunum measured about 10.73 µm in diameter and were localised between the basal pole and the nucleus of the host cells. This particular localization contributed to the nuclear displacement (Fig. 3). Similar degree and patterns of mucosal inflammation, characterised by a transmural infiltration of mononuclear cells, were observed also in the colonic and caecal walls. Large areas of epithelial necrosis were found associated with the thickened wall. In examined birds, the caecum was the intestinal site where the highest degree of severity was scored for histopathological lesions (Table 1 and 3; Fig. 4). An extra-epithelial *Eimeria* localisation was also observed, mainly in the colon. In fact, in this intestinal segment free microgamonts, intra-macrophagic merozoites and several schizonts were found interspersed in the lamina propria. An opposite intraepithelial localization was observed for macrogamonts found in the caecal and colonic mucosa respect to those found in the small intestine. Indeed, they were located between the nucleus and the free pole of the host cells (Fig. 5). Moreover, these macrogamonts were larger (about 15.25 µm in diameter) than those observed in the small intestine.

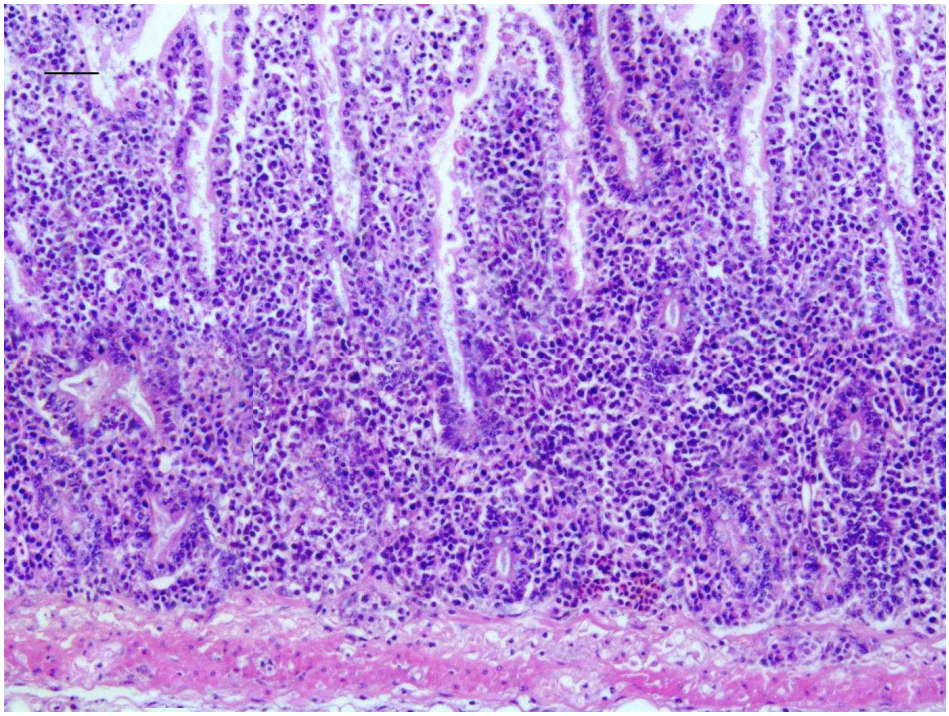

Fig. 2: Second half of the small intestine infected by *Eimeria legionensis*: severe displacement of the deep crypts, (HE, 20X). Scale bar: 500 µm

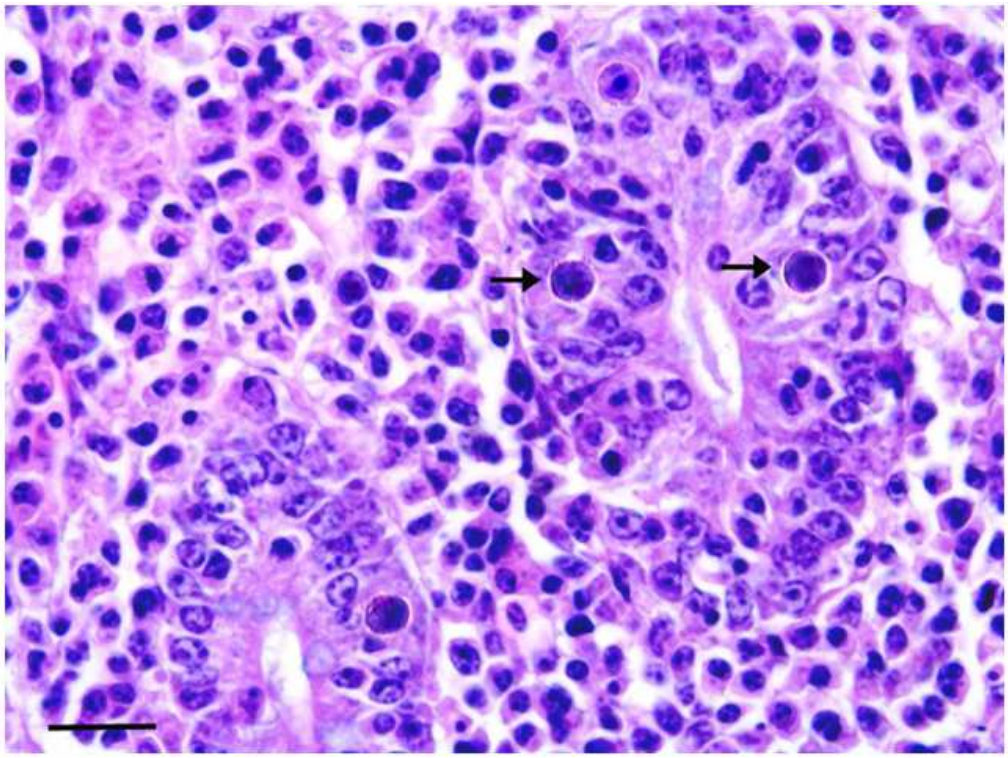

Fig. 3: Terminal portion of the small intestine: intraepithelial *Eimeria kofoidi* macrogamonts localised between the basal pole and the nucleus of the infected host cells, (HE, 100X). Scale bar: 50 µm

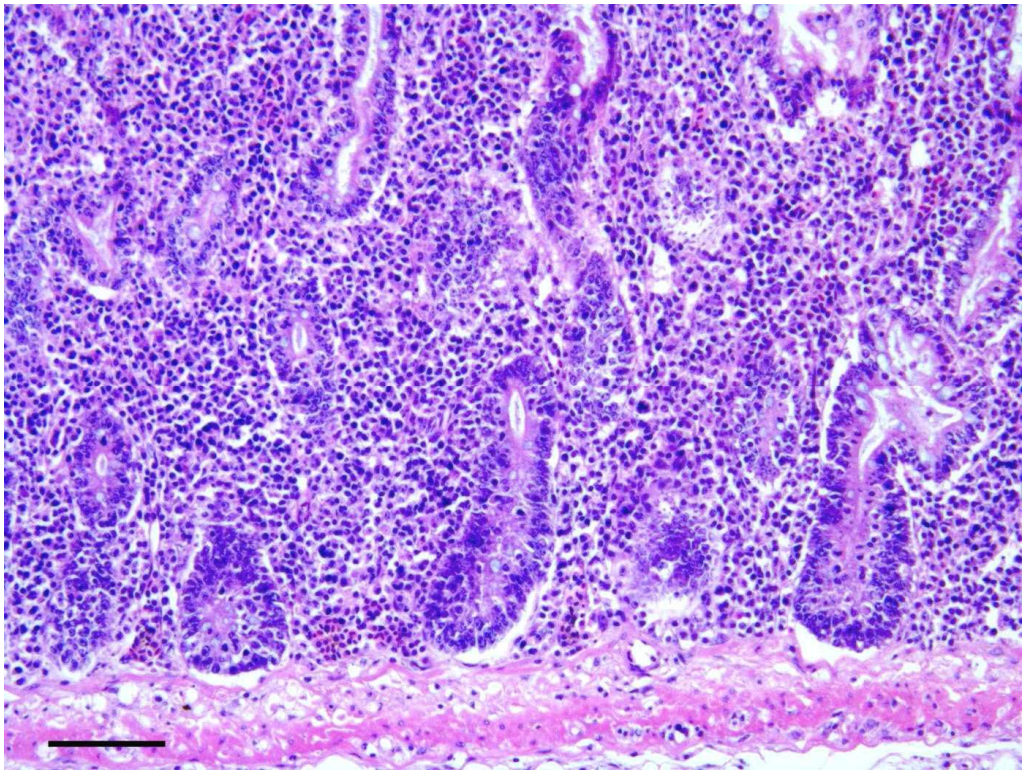

Fig. 4: Transmural infiltration of mononuclear cells in the caecum, (HE, 20X). Scale bar: 500 µm

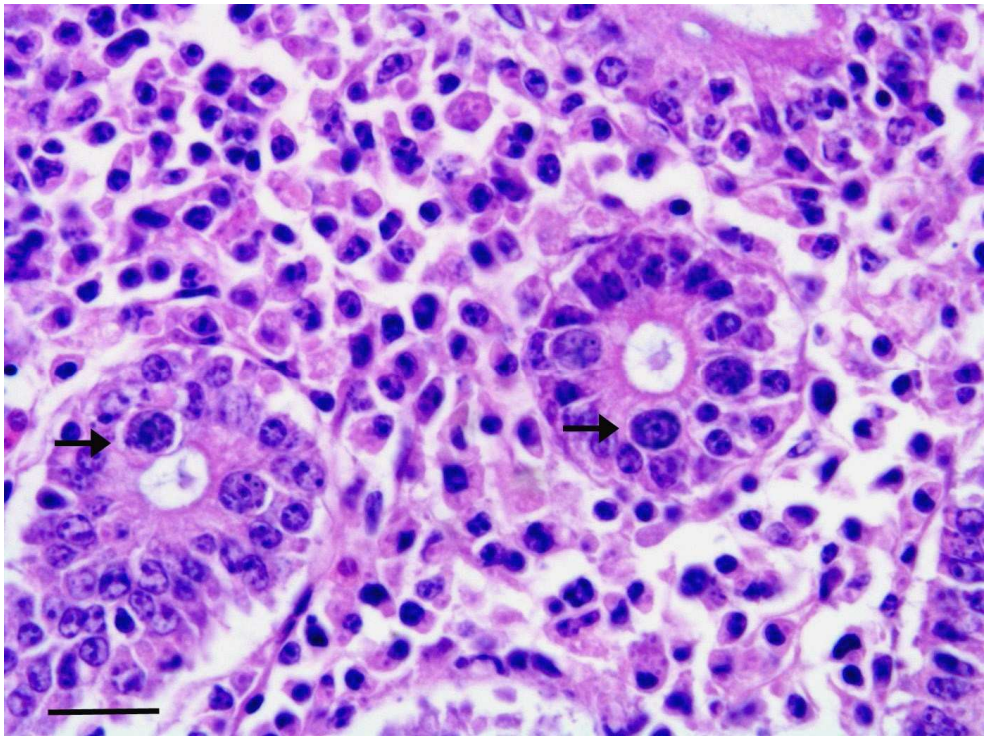

Fig. 5: Caecal mucosa: intraepithelial *Eimeria legionensis* macrogamonts localised between the nucleus and the free pole of the host cells, (HE, 100X). Scale bar: 50 μm

Table 3: Range and the mean lesion score values found in the different tracts of the small and the large intestines taken by deceased red-legged partridges (*Alectoris rufa*) found infected by *Eimeria kofoidi* and *Eimeria legionensis*

	Infection score*					Lesion score**				
Sample	Duodenum	Jejunum	Ileum	Caecum	Colon	Duodenum	Jejunum	Ileum	Caecum	Colon
1	0	2	3	3	2	0	2	3	4	1
2	0	1	3	2	1	0	1	4	4	0
3	0	1	3	3	2	0	0	3	3	1
4	0	1	3	3	2	0	2	3	3	1
5	0	1	3	3	2	0	1	4	4	1
6	0	1	3	2	2	0	2	4	4	1
7	0	2	3	3	2	0	1	3	3	1
8	0	1	3	3	2	0	0	3	4	1
9	0	1	3	2	1	0	2	4	3	0
10	0	1	3	3	2	0	1	3	4	1
11	0	2	3	3	2	0	0	4	4	1
12	0	1	3	3	2	0	3	4	3	0
13	0	1	3	3	2	0	2	3	4	1
14	0	2	3	3	2	0	1	3	3	1
15	0	2	3	3	2	0	0	3	3	1
16	0	1	3	3	2	0	2	4	4	1
17	0	1	3	2	1	0	1	3	3	0
18	0	1	3	3	2	0	0	3	4	1
19	0	2	3	3	2	0	0	3	3	1
20	0	1	3	3	2	0	2	3	4	1
21	0	2	3	3	2	0	2	4	3	1
22	0	1	3	2	1	0	1	3	4	0
23	0	2	3	3	2	0	2	4	4	1
24	0	1	3	2	2	0	1	4	4	1

Table 3: Continue

25	0	1	3	3	2	0	0	3	3	1
26	0	1	3	2	1	0	1	3	4	0
27	0	1	3	3	2	0	2	4	3	1
28	0	2	3	3	2	0	1	3	4	1
29	0	1	3	2	2	0	2	4	4	1
30	0	2	3	3	2	0	2	4	3	1
31	0	1	3	3	1	0	1	3	4	0
32	0	2	3	3	2	0	2	3	4	1
33	0	1	3	3	2	0	1	4	4	1
34	0	2	3	3	2	0	2	3	3	1
35	0	1	3	3	1	0	1	3	4	0
36	0	2	3	3	2	0	1	4	4	1
37	0	2	3	3	2	0	1	4	4	1
38	0	1	3	3	2	0	0	4	4	1
39	0	2	3	3	2	0	1	3	4	1
40	0	1	3	2	1	0	2	4	4	0
41	0	2	3	3	2	0	1	3	3	1
42	0	1	3	3	2	0	0	3	3	1
43	0	2	3	3	1	0	1	4	3	1
44	0	2	3	3	2	0	1	3	3	1
45	0	2	3	2	2	0	1	3	3	1
46	0	1	3	3	1	0	1	3	4	0
47	0	1	3	3	2	0	2	4	3	1
48	0	2	3	3	2	0	1	3	4	1
49	0	1	3	3	2	0	1	3	4	1
50	0	2	3	3	2	0	1	4	3	1
Mean	**0.0**	**1.4**	**3.0**	**2.8**	**1.8**	**0.0**	**1.2**	**3.4**	**3.6**	**0.8**
SD*	**0**	**0.50**	**0.00**	**0.40**	**0.40**	**0.00**	**0.75**	**0.50**	**0.50**	**0.40**

***SD:** Standard Deviation

Discussion

Among the several coccidian species described in the genus *Alectoris*, including *A. rufa*, *E. legionensis* and *E. kofoidi* have been frequently reported as responsible for coccidiosis outbreaks (Pellérdy, 1974; Levine, 1988; Naciri *et al*., 2011). Also in northern Italy, these two species were reported as the cause of outbreaks of coccidiosis in farmed *A. rufa* (Bolognesi *et al*., 2006).

In previous literature, there is a full agreement on *E. legionensis* oocyst dimensions (Cordero Del Campillo and Pla Hernàndez, 1966; Bolognesi *et al*., 2006; Naiciri *et al*., 2011), while this is not the case of *E. kofoidi* oocysts. Indeed, in the original description by Yakimoff and Matikaschwili (1936) in chukka partridges (*Alectoris chukar*) and grey partridges (*Perdix perdix*), the average size of *E. kofoidi* oocysts is of 20×17.6 μm. However, as in the present study, smaller mean dimensions have been reported for *E. kofoidi* oocysts in *A. rufa* (Lizcano Herrera and Romero Rodriguez, 1972; Bolognesi *et al*., 2006; Naciri *et al*., 2011).

Results from the gross and histopathological examination of the intestines are indicative that *E. kofoidi* and *E. legionensis* coccidiosis was the main cause for the death of the 50 red-legged partridges examined in this study, confirming the epidemic-like coccidiosis outbreaks with considerable mortality previously reported in this bird species (Bolognesi *et al*., 2006; Naciri *et al*., 2011). Indeed, the severity of gross and histopathological lesions found in this study in infected intestinal segments are similar to histopathological lesions described in other galliform birds infected by coccidia (Long, 1973; Conway *et al*., 1990). In particular, in these previous reports clinical coccidiosis has been found associated with thickened intestinal wall, loss of epithelial tissues and villi, necrosis, edema and erosion of the sub-mucosa, glandular tissue cells infiltration, decreased villar height and marked hyperplasia of lymphoid cells. Mononuclear cells infiltration in the intestinal mucosa was a salient finding in intestinal coccidiosis by *Emeria garnhami* in *Coturnix coturnix* (Rasheda and Bano, 1985).

In previous studies, some disagreements are also present with regard to the localisation of *E. kofoidi* and *E. legionensis*. In particular, in the redlegged partridge *E. kofoidi* was found located in the terminal portion of ileum and in the caecum by Litzcano Herrera and Romero Rodriguez (1972) and in the small intestine, mainly in the duodenal loop and jejunum but also in ileum, by Naciri *et al*. (2011). In the deceased red-legged partridges here examined, *E. kofoidi* was found localised

in the jejunum and ileum both at parasitological and histological analysis, and nor the duodenum nor the caecum were found infected. *E. legionensis* was described for the first time in Spain in *A. rufa* and it was considered responsible for caecal infections (Cordero del Campillo and Pla-Hernandez, 1966). This same localisation for *E. legionensis*, has been recently reported by Naciri *et al*. (2011). However, data from these previous studies are not based on histopathological analysis of infected intestinal segments.

Histological and morphological analysis, here reported, evidenced that *E. kofoidi* infect the small intestine, i.e. jejunal and ileal segments, while *E. legionensis* localises in the caecum and colon. In addition, findings from this study showed that *E. kofoidi* macrogamonts are localised between the basal pole and the nucleus of infected cells and this particular localization contributed to nuclear displacement. Macrogamonts of *E. legionensis* are instead found between the nucleus and the free pole of the host cells. In *Eimeria* infected red-legged partridges, morphological oocysts examination supported by the evaluation of gross lesions and by the different localisation in the intestine are considered helpful tools for species identification (Naciri *et al*., 2011).

Gross and histopathological lesions here found in *E. kofoidi* and *E. legionensis* infected birds may explain the weight loss, the poor feed conversion and the high mortality reported for partridge coccidiosis in previous studies (Reck and McQuistion, 1994; Bolognesi *et al*., 2006; Naciri *et al*., 2011), and confirm the frequent involvement of these two *Eimeria* species in coccidiosis outbreaks of *A. rufa*.

Conclusion

Results here obtained indicate the colon as a further localisation site of *E. legionensis*, and provide new data on intestinal lesions and intracellular localisation of *E. kofoidi* and *E. legionensis* in *A. rufa*. In particular, findings from this study concerning the different size of *E. kofoidi* and *E. legionensis* macrogamonts and their different localisation in the intestine and within the host cells, could give a further help for the differentiation of these two coccidian species in deceased birds.

Author Contributions

G. Fichi: Partecipated in parasitological analysis and gross examination, contributed to the interpretation of the results, drafting and revision of the manuscript.

G. Rossi: Partecipated in histopathological analysis and contributed to the to the interpretation of the results, drafting and revision of the manuscript.

Stefania Perrucci: Concepted and designed the study, contributed to the acquisition of parasitological data, interpretation of the results, drafting and revision of the manuscript.

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Conflict of Interest Statement

None of the authors has any financial, personal or other associations that may influence the content of the paper.

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