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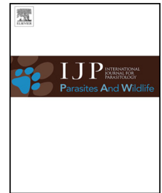

Molecular survey on the occurrence of avian haemosporidia, *Coxiella burnetii* and *Francisella tularensis* in waterfowl from central Italy

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ABSTRACT

The aim of the present study was to evaluate the occurrence of some avian Haemosporidia, *Coxiella burnetii* and *Francisella tularensis* in waterfowl from Tuscany wetlands. One-hundred and thirty-three samples of spleen were collected from regularly hunted wild birds belonging to 13 different waterfowl species. DNA extracted from each sample was submitted to PCR assays and sequencing to detect the pathogens. Thirty-three samples (24.81%) were positive with PCR for at least one pathogen: 23 (17.29%) for *Leucocytozoon* spp., 6 (4.51%) for *Plasmodium* spp., 4 (3%) for *C. burnetii*, 2 (1.5%) for *Haemoproteus* spp. No specific *F. tularensis* amplifications (0%) were detected. To the best of our knowledge, this study firstly reports data about haemosporidian and *C. burnetii* infections in waterfowl from Italy.

1. Introduction

Avian haemosporidia are a group of protozoan parasites, among which *Plasmodium, Haemoproteus* and *Leucocytozoon* genera are encountered. These parasites are transmitted by blood sucking dipteran vectors, with birds acting as intermediate hosts (Valkiunas, 2005). In detail, after the inoculation of sporozoite stages by the vectors during the bloodmeal, exoerytrhrocytic schizonts develop in different tissues (i.e. spleen, liver, lungs, heart, brain …) giving gametocytes into the blood cells. These latter stages are infective for the vectors.

Haemosporidians occur worldwide except in Antarctica (Beadell et al., 2009) and most infections are relatively benign, probably due to long-term host parasites evolutionary associations (Bennet et al., 1993). The occurrence of these protozoa has been reported from several birds' species (Schmid et al., 2017; Dimitrov et al., 2018; Ferreira-Junior et al., 2018; Valkiunas et al., 2019; Schumm et al., 2019) and from dipteran species (Ionică et al., 2017; Žiegytė et al., 2017; Martin et al., 2019; Schoener et al., 2019).

Domestic *Anatidae* are highly susceptible to haemosporidia (Valkiunas, 2005) and wild waterfowl have been reported as infected in several countries (Loven et al., 1980; Reeves et al., 2015; Ramey et al., 2015; Meixell et al., 2016).

To the best of our knowledge, a unique survey by Sacchi and Prigioni (1986) evaluating occurrence of haemosporidia in wild waterfowl by microscopy in Italy yielded no positive results.

Coxiella burnetii is the etiologic agent of Q fever, a worldwide zoonotic bacterial disease. Even though domestic ruminants are considered as the main reservoirs for this pathogen, several wild mammals have been found to be hosts of this microorganism. *C. burnetii* has been found in birds (Babudieri and Moscovici, 1952; Stein and Raoult, 1999; Ebani et al., 2016), too, but little is known about the transmission patterns among avian populations. Animals as well as humans usually become infected through oral and inhalation routes, but the transmission of *C. burnetii* is also possible via tick bites (Porter et al., 2011).

Francisella tularensis is the causative agent of the severe zoonotic disease tularemia. It is a Gram negative bacterium, with a wide range of hosts including invertebrates and mammals. Birds have been experimentally infected with *F. tularensis* (Mörner and Mattsson, 1988) and naturally acquired infections have been reported in different avian species (Green and Wade, 1929; Green and Shillinger, 1932; Nakamura, 1950; Stahl et al., 1969; Mörner and Mattsson, 1983). However, studies about tularemia in avian populations were conducted many years ago and updated information is not available. Moreover, symptoms and lesions in birds are not known and some authors suggested that these animals may acquire the infection without developing disease (Mörner and Mattsson, 1988).

The transmission of *F. tularensis* may occur through haematophagous arthropods, as well as through direct or indirect contact with

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Table 1
PCR primers and conditions employed in the assays for the detection of each pathogen.

Pathogens	Amplicons (target gene)	Primers sequence (5' – 3')	PCR conditions	References
* *Haemoproteus* spp. *Plasmodium* spp. *Leucocytozoon* spp.	617 bp (cytochrome b)	HAEMNFI (CATATATTAAGAGAATTATGGAG) HAEMNR3 (ATAGAAAGATAAGAAATACCATTC)	94 C - 30 s 50 C–30 s 72 C–45 s (20 cycles)	[Hellgren et al., 2004]
** *Haemoproteus* spp. *Plasmodium* spp.	480 bp (cytochrome b)	HAEMF (ATGGTGCTTTCGATATATGCATG) HAEMR2 (GCATTATCTGGATGTGATAATGGT)	94 C- 30 s 50 C–30 s 72 C–45 s (35 cycles)	[Bensch et al., 2000]
** *Leucocytozoon* spp.	478 bp (cytochrome b)	HAEMFL (ATGGTGTTTTAGATACTTACATT) HAEMR2L (CATTATCTGGATGAGATAATGGTGC)	94 C- 30 s 50 C–30 s 72 C–45 s (35 cycles)	[Hellgren et al., 2004]
Coxiella burnetii	687 bp (IS1111a)	Trans-1 (TATGTATCCACCGTAGCCAGT) Trans-2 (CCCAACAACACCTCCTTATTC)	95 °C–30 s 64 °C–1 min 72 °C–1 min	[Berri et al., 2009]
Francisella tularensis	400 bp (TUL4)	TUL4-435 (TCGAAGACGATCAGATACCGTCG) TUL4-863 (TGCCTTAAACTTCCTTGCGAT)	96 °C–1 min 60.5 °C–1 min 72 °C–1 min	[Milutinović et al., 2008]

*Primary amplification; ** Secondary amplification.

Table 2
Number of specimens and detail of positive reactions for *Coxiella burnetii, Haemoproteus* spp., *Leucocytozoon* spp. and *Plasmodium* spp. in relation to the tested avian species.

Animal species	No. tested specimen	Positive reactions
Anseriformes		
Anas crecca	63	15 *Leucocytozoon* spp. 3 *C. burnetii* 2 *Plasmodium* spp.
Anas platyrhynchos	21	1 *Haemoproteus* spp.
Anas penelope	19	3 *Leucocytozoon* spp. 4 *Plasmodium* spp. 1 *C. burnetii*
Anas clypeata	10	2 *Leucocytozoon* spp.
Anas acuta	3	2 *Leucocytozoon* spp.
Aythya ferina	3	Negative
Tadorna tadorna	2	Negative
Anas strepera	2	1 *Leucocytozoon* spp.
Anas querquedula	1	Negative
Aythya fuligula	1	Negative
Anser anser	1	Negative
Charadriiformes		
Gallinago gallinago	6	1 *Haemoproteus* spp.
Gruiformes		
Fulica atra	1	Negative
Total	133	35

infected animals.

In view of the scant data available about the spreading of Haemosporidia, *C. burnetii* and *F. tularensis* among avian population in Italy, the aim of the present study was to investigate the occurrence of these pathogens among wild waterfowl hunted in Tuscany (Central Italy) wetlands.

2. Material and methods

2.1. Animals

The study was performed on 133 wild birds belonging to 13 different waterfowl species of the Orders *Anseriformes*, *Charadriiformes* and *Gruiformes*. More in detail, the analysis were executed on 63 common teals (*Anas crecca)*, 21 mallards (*Anas platyrhynchos*), 19 eurasian wigeons (*Anas penelope*), 10 northern shovelers (*Anas clypeata*), 6 common snipes (*Gallinago gallinago*), 3 pintails (*Anas acuta*), 3 common pochards (*Aythya ferina)*, 2 common shelducks (*Tadorna tadorna*), 2 gadwalls (*Anas strepera*), 1 garganey (*Anas querquedula*), 1 tufted duck (*Aythya fuligula*), 1 greylag goose (*Anser anser*), and 1 eurasian coot (*Fulica atra*).

All animals were regularly hunted during the 2016 and 2017 hunting seasons (September–January) in the Province of Pisa, in the wetland "Padule di Fucecchio" (43°48′N 10°48′E/43.8°N 10.8°E43.8), located on the migration route of waterfowls. All the animals were intended for human consumption, so the carcasses were immediately refrigerate until processing (about within 24 h). Bird's carcasses were submitted to assessment of body condition and examined for ectoparasites. Viscera were removed from each bird, maintained at 4 °C and sent to the laboratories. Impression smears were prepared from spleen of each animal for microscopical examination. The remaining portions were stored at −20 °C for molecular analysis. It was not possible to obtain blood specimens to investigate blood stage parasites and sellers allowed us to collect spleens, only. However this tissue was reported as sensitive in detecting haemosporidian parasites (Scaglione et al., 2016; Valkiunas and Iezhova, 2017). Spleen allows the detection of *C. burnetii* and *F. tularensis*, too, because hematogenous spread of these bacteria lead to involvement of several organs including this one (Bell, 1980; Maurin and Raoult, 1999).

2.2. Microscopical examination

The spleen impression smears were prepared on glass slide, fixed in methanol and stained with modified Wright's solution. Each smear was examined through 100 fields at low magnification (400X), then at least 100 fields were observed at high magnification (1000X).

2.3. Molecular analysis

Extraction of total DNA was carried out from up to 10 mg of each spleen sample using the DNeasy® Blood & Tissue kit (QIagen, Milano, Italy) and following the manufacturer's instructions. DNA specimens were stored at 4 °C until used as template for the PCR assays.

Different PCR protocols were carried out to detect DNA of pathogens. *Haemoproteus* spp., *Plasmodium* spp., *Leucocytozoon* spp. DNA were detected, with a nested PCR protocol, according to Hellgren et al. (2004) and Bensch et al. (2000). The extracted DNA was first subjected to an amplification common to *Leucocytozoon*, *Haemoproteus* and *Plasmodium*, and then, its product was submitted to two parallel PCRs, one amplifying both *Haemoproteus* and *Plasmodium* while the another one amplifying *Leucocytozoon* only.). *C. burnetii* and *F. tularensis* DNA were investigated, using primers and procedures previously described, respectively (Milutinović et al., 2008; Berri et al., 2009). Target genes, primers sequences and PCR conditions are summarized in Table 1.

PCR amplifications were performed using the EconoTaq PLUS 2x Master Mix (Lucigen Corporation, Middleton, Wisconsin, USA) and an automated thermal cycler (Gene-Amp PCR System 2700, Perkin Elmer, Norwalk, Connecticut, USA).

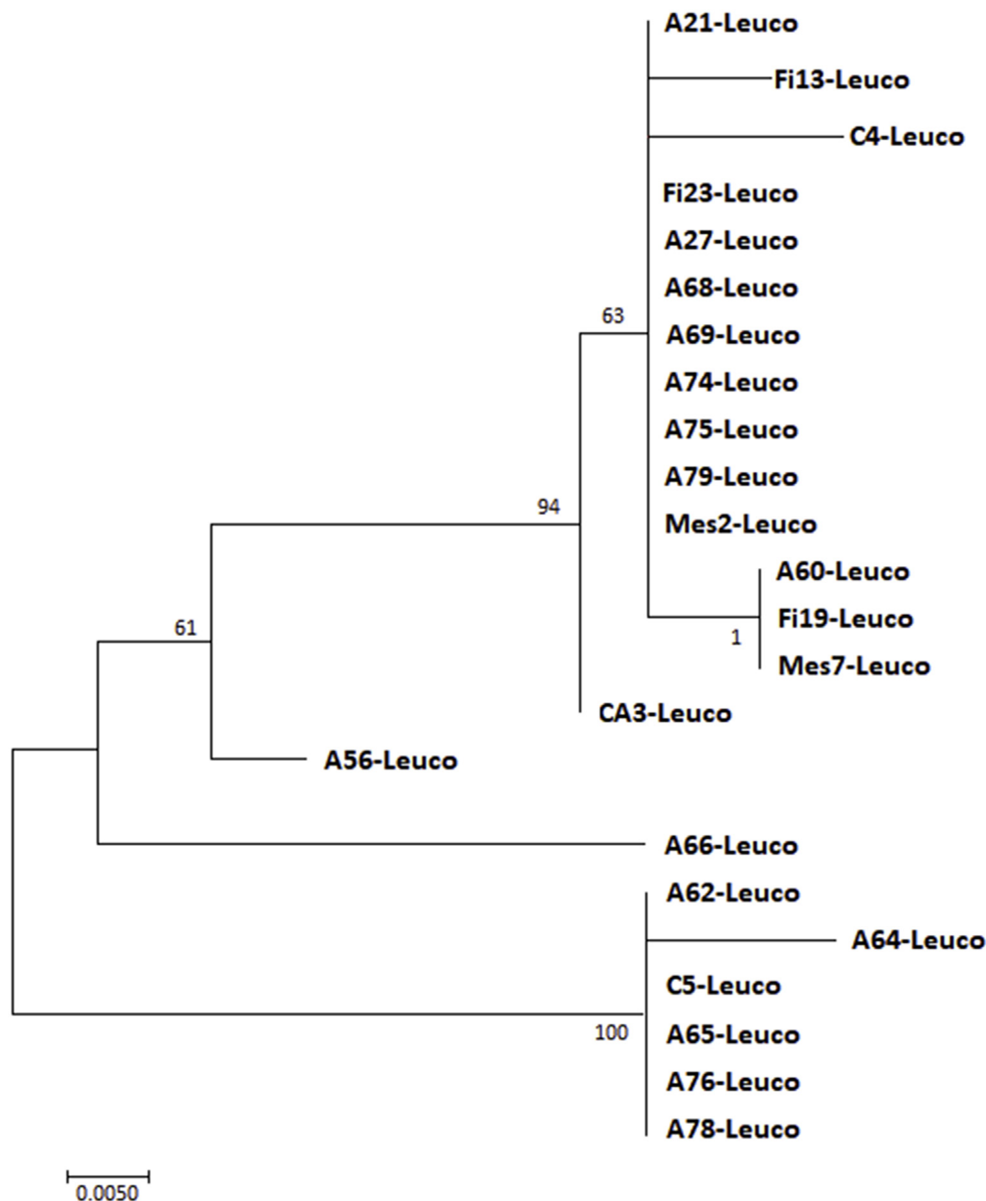

Fig. 1. Phylogenetic tree showing the *Leucocytozoon* sequencing results.

PCR products were analysed by electrophoresis on 1.5% agarose gel at 100 V for 45 min; gel was stained with ethidium bromide and observed. SharpMass™ 100 Plus Ladder (Euroclone, Milano, Italy) was used as DNA marker.

Samples resulted positive for *Haemoproteus* spp., *Plasmodium* spp. and *Leucocytozoon* spp. were sequenced on both strands by the commercial laboratory BMR-Genomics (Padua, Italy), using the inner primers. The sequence obtained was assembled and corrected by visual analysis of the electropherogram using Bioedit v.7.0.233 and compared with those available in GenBank ® 4 using the BLASTn ® program (http://www.ncbi.nlm.nih.gov/BLAST). Sequences obtained by overlapping both sense and antisense strands were exclusively used in the article.

3. Results

All animals of the study were in good nutrition conditions and no lesions and ectoparasites were observed during the carcasses' manipulation. No microorganisms or parasite stages were observed in smears.

Among the 133 tested samples, 33 (24.81%) were PCR positive for at least one pathogen. More in details, 23 (17.29%) birds were positive for *Leucocytozoon* spp., 6 (4.51%) for *Plasmodium* spp., 4 (3%) for *C. burnetii*, 2 (1.5%) for *Haemoproteus* spp. No specific *F. tularensis* amplifications (0%) were detected (Table 2).

Sequence analysis for *Leucocytozoon* spp. revealed that the majority of the isolates fell in two broad groups.

One group (here called Leucocytozoon "lineage duck 1") was composed of 9 isolates, showing 100% homology with parasites sequenced from previous studies (L. Kvleu MG593842; L; L. TS-2014a voucher MN-08-A-0235, KJ577823; L. MMSL02, KU295418; L. TUSW04 JQ314223).

The other group (Leucocytozoon "lineage duck 2") was composed of 6 isolates, exhibiting 100% homology with different parasites sequenced (DUCK 18, KM386326; L. BWTE20, KU363710).

Six isolates showed 99% homology with haplotype duck 1, with two mismatches maximum.

One isolate exhibited 100% identity with L. DUCK40 KM386348.

One isolate exhibited 98% homology with L.duck. Duck32 KM386340 e Duck34 KM386342. The phylogenetic tree in Fig. 1 illustrates the leucocytozoon sequencing results.

Table 3
–Sequencing analysis results of the samples resulted PCR positive for Haemosporidia.

Host species	Parasite genus	Number bp	% homology	GenBank sequences
Anas crecca	*Leucocytozoon*	514	100	L.Kvleu; L.TS-2014a vouch MN-08B-0298; L.TS-2014°vouch MN-08-A-0235; L.MMSL02; TUSW04
Anas crecca	*Leucocytozoon*	501	100	L.DUCK18; L.BWTE20
Anas crecca	*Leucocytozoon*	344	100	L.DUCK18; L.BWTE20
Anas crecca	*Leucocytozoon*	504	100	L.DUCK18; L.BWTE20
Anas crecca	*Leucocytozoon*	518	100	L.DUCK40
Anas crecca	*Leucocytozoon*	444	100	L.Kvleu; L.TS-2014a vouch MN-08B-0298; L.TS-2014°vouch MN-08-A-0235; L.MMSL02; TUSW04
Anas crecca	*Leucocytozoon*	511	100	L.Kvleu; L.TS-2014a vouch MN-08B-0298; L.TS-2014°vouch MN-08-A-0235; L.MMSL02
Anas crecca	*Leucocytozoon*	466	100	L.Kvleu; L.TS-2014a vouch MN-08B-0298; L.TS-2014°vouch MN-08-A-0235; L.MMSL02; TUSW04
Anas crecca	*Leucocytozoon*	439	100	L.Kvleu; L.TS-2014a vouch MN-08B-0298; L.TS-2014°vouch MN-08-A-0235; L.MMSL02; TUSW04
Anas crecca	*Leucocytozoon*	443	100	L.DUCK18; L.BWTE20
Anas crecca	*Leucocytozoon*	515	100	L.DUCK18; L.BWTE20
Anas crecca	*Leucocytozoon*	507	100	L.Kvleu; L.TS-2014a vouch MN-08B-0298; L.TS-2014°vouch MN-08-A-0235; L.MMSL02; TUSW04
Anas acuta	*Leucocytozoon*	495	100	L.DUCK18; L.BWTE20
Anas penelope	*Leucocytozoon*	502	100	L.Kvleu; L.TS-2014a vouch MN-08B-0298; L.TS-2014°vouch MN-08-A-0235; L.MMSL02; TUSW04
Anas clypeata	*Leucocytozoon*	518	100	L.Kvleu; L.TS-2014a vouch MN-08B-0298; L.TS-2014°vouch MN-08-A-0235; L.MMSL02; TUSW04
Anas clypeata	*Leucocytozoon*	496	100	L.Kvleu; L.TS-2014a vouch MN-08B-0298; L.TS-2014°vouch MN-08-A-0235; L.MMSL02; TUSW04
Anas crecca	*Leucocytozoon*	509	99	L.Kvleu; L.TS-2014a vouch MN-08B-0298; L.TS-2014°vouch MN-08-A-0235; L.MMSL02; TUSW04; TUSW03
Anas crecca	*Leucocytozoon*	508	99	L.Kvleu; L.TS-2014a vouch MN-08B-0298; L.TS-2014°vouch MN-08-A-0235; L.MMSL02; TUSW04; TUSW03
Anas acuta	*Leucocytozoon*	496	99	L.Kvleu; L.TS-2014a vouch MN-08B-0298; L.TS-2014°vouch MN-08-A-0235; L.MMSL02; TUSW04; TUSW03
Anas strepera	*Leucocytozoon*	503	99	L.Kvleu; L.TS-2014a vouch MN-08B-0298; L.TS-2014°vouch MN-08-A-0235; L.MMSL02; TUSW04; TUSW03
Anas penelope	*Leucocytozoon*	489	99	L.Kvleu; L.TS-2014a vouch MN-08B-0298; L.TS-2014°vouch MN-08-A-0235; L.MMSL02; TUSW04; TUSW03
*Anas penelope**	*Leucocytozoon*	355	99	L.Kvleu; L.TS-2014a vouch MN-08B-0298; L.TS-2014°vouch MN-08-A-0235; L.MMSL02; TUSW04; TUSW03
Anas crecca	*Leucocytozoon*	455	98	LDUCK32; LDUCK34
Anas crecca	*Plasmodium*	527	100	*Plasmodium circumflexum*
Anas crecca	*Plasmodium*	64	100	*Plasmodium polare*
Anas penelope	*Plasmodium*	290	100	*Plasmodium circumflexum*
Anas penelope	*Plasmodium*	462	100	P.Sybor2
*Anas penelope**	*Plasmodium*	421	100	*Plasmodium circumflexum*
Anas penelope	*Plasmodium*	145	99	P. Sybor2
Gallinago gallinago	*Haemoproteus*	497	100	H230
Anas platyrhynchos	*Haemoproteus*	478	100	H230

Legend - *: one *Anas penelope* scored positive to *Leucocytozoon* and *Plasmodium circumflexum*.

Table 4
PCR results for pathogen, lineage and host species. In each square it is indicated the number of animal scored positive for each pathogen with the percentage of sequence homology.

Animal species	L. "lineage duck 1"	L. "lineage duck 2"	L. duck 40	L. duck 32	*P. polare*	*P. circumflexum*	P. sybor 2	H 230
Anas crecca	**6 (100%)** **2 (99%)**	**5 (100%)**	**1 (100%)**	**1 (98%)**	**1 (100%)**	**1 (100%)**		
Anas acuta	**1 (99%)**	**1 (100%)**						
Anas penelope	**2 (100%)** **1(99%)**					**2 (100%)**	**1 (100%)** **1 (99%)**	
Anas clypeata	**2 (100%)**							
Anas streptera	**1 (99%)**							
Gallinago gallinago								**1 (100%)**
***Anas** platyrhynchos*								**1 (100%)**

Sequences analysis for *Plasmodium* found 100% homology with *Plasmodium circumflexum* for 3 samples and *Plasmodium polare* for one sample; one specimen scored 100% similar to P. Sybor2 isolate. One sample showed 99% homology with the sequence P. Sybor2. Two amplicons were identified as *Haemoproteus* spp.

A mixed infection by *Leucocytozoon* and *P. circumflexum* was observed in one *A. penelope* whereas a mixed infection by *Leucocytozoon* and *C. burnetii* was detected in one *A. crecca*.

More detailed data about hosts and agents are reported in Tables 3 and 4.

4. Discussion

The investigated subjects showed a 23.3% prevalence of infection by haemosporidia, with *Leucocytozoon* being the most abundant parasite detected via PCR (17.29%). In similar investigations this genus was the more frequently encountered in *Anseriformes*, both domestic (Ramey et al., 2012) and wild (Ramey et al., 2015; Reeves et al., 2015; Smith et al., 2016; Seimon et al., 2016). The L. "lineage duck 1" showed 100% identity with parasites detected from *Anser domesticus* (L. Kvleu MG593842, Turkey), *Anser indicus* (L. TS-2014a voucher MN-08-A-0235, KJ577823, Mongolia) *A. platyrhynchos* (L. MMSL02, KU295418, Alaska) and *Cygnus colombianus* (L. TUSW04 JQ314223, Alaska). The "lineage duck 2" exhibited 100% identity with isolates coming from *Anas* sp.(L. DUCK 18, KM386326, Pacific America) and *Anas discors* (L. BWTE20, KU363710, North America). These findings suggest that the lineages observed in the present study are well adapted to *Anatidae* birds. Additionally, since the same lineages were observed also in other continents, the isolates described in Italy don't seem to be confined only in Europe. Such findings needs further studies to strengthen this hypothesis.

Plasmodium was identified in 6 animals (4.51%), 2 *A. crecca* and 4 *A. penelope.* In these animals 2 species and different sequences were recognized. This haemosporidian genus, in contrast with *Leucocytozoon* and *Haemoproteus*, is not considered as a host specific parasite (Reeves et al., 2015; Smith et al., 2016). *P. circumflexum* has been recorded to have a low host specificity (Dimitrov et al., 2015), and in the present study was reported in a mixed infection with *Leucocytozoon. P. polare*

was identified in one specimen from *A. crecca.* This haemosporidian species has been previously isolated from great tits (*Parus major*), from Switzerland (Rooyen et al., 2013). *Haemoproteus* was identified only in 2 samples (1.5%), from a *G. gallinago* and a *A. platyrhynchos;* however, isolated sequences did not allow us to recognize the species.

Data from Italy are scanty and consist of a study, carried out by microscopy, in which *Leucocytozoon* and *Haemoproteus* were not detected in *Anatidae* and *Charadridae* from North Italy (Sacchi and Prigioni, 1986). At the best of our knowledge, this is the first report of haemosporidian infection in *A. penelope, A. strepera, A. querquendula, T. tadorna* and *G. gallinago.*

Similar studies have been conducted on waterfowl in the USA (Ramey et al., 2015; Reeves et al., 2015; Meixell et al., 2016) in a lower number of bird species and in injured birds from Japan (Inumaru et al., 2017). Nevertheless, the above mentioned authors reported higher prevalence values in respect to the present study. Different results obtained in literature could be due to both extrinsic and intrinsic factors, such as methods used. The season in which the study is performed is important, because in dry environment the prevalence of infection would be lower (Smith and Ramey, 2015), even if in the present study sampling was conducted during autumn/winter seasons. The present investigation detected four animals (3 *A. crecca* and 1 *A. penelope*) positive (3%) for *C. burnetii*, whereas no *F. tularensis*-positive reactions were found. *C. burnetii* and *F. tularensis* may be transmitted by haematophagous arthropods, mainly ticks, but other transmission routes are possible. *C. burnetii*-positive birds could have contracted the pathogen by ticks' bite, but it is easier to consider the oral route. In fact, waterfowl may travel large distances per day and reach areas where infected animals, wild and domestic, are present. Birds infected by *C. burnetii* play a relevant epidemiological role, because they can disperse the bacteria in the environment through their feces and they may be source of coxiellae for ticks during the blood meal.

On the basis of previous epidemiological studies in mammals and data relative to human infections, *F. tularensis* seems to not be largely present in Italy (Pascucci et al., 2015; Ebani et al., 2016, 2017; Graziani et al., 2016; Rocchigiani et al., 2018), thus the negative results of the tested waterfowl could reflect the true epidemiological status. *F. tularensis* has been proven to be able to infect different avian species, thus birds could be cause of infection for humans. Hunters are more at risk of infection having direct or indirect contact with wild birds, as suggested by Padeshki et al. (2010), who reported a case of tularemia with typical ulceroglandular form in a hunter who acquired the infection through a nail scratch from a buzzard (*Buteo buteo*).

Data about the transmission of *C. burnetii* and *F. tularensis* among avifauna are scant in scientific literature. Two studies have been carried out on ticks collected from birds in Italy: Toma et al. (2014) detected *C. burnetii* DNA in 42/127 analysed ticks collected from migratory birds of different species, but not waterfowl; Pajoro et al. (2018) did not detected *C. burnetii* and *F. tularensis* in any ticks collected from 124 wild birds. A recent investigation has been carried out on fecal samples collected from 673 migratory birds along the Mediterranean – Black Sea flyway and bacteriological and molecular analyses found neither *F. tularensis* nor *C. burnetii* (Najdenski et al., 2018).

5. Conclusions

The present study firstly reports data about haemosporidia and *C. burnetii* infections in waterfowl from Italy. In particular, these findings would add more data about haemosporidia species and strains circulating in avian population. Moreover, it has been confirmed the involvement of wild birds in the epidemiology of *C. burnetii,* suggesting that these animals may be direct and indirect source of infection for people. In fact, humans could be at risk when manipulating infected live birds or carcasses, as well as they could contract the infection because of the environment contamination with coxiellae excreted through birds' droppings.

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Conflicts of interest

All authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence their work.

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