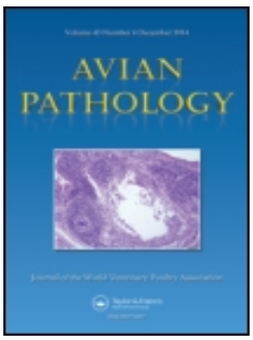

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Sequence subtyping of *Trichomonas gallinae* from Bonelli's eagle (*Aquila fasciata*) during four years (2014-2017) reveals that MLS type is associated with lesions

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ABSTRACT:

Avian trichomonosis is a parasitic disease that affects wild birds, The objective of this work was to determine the importance of avian trichomonosis in Bonelli's eagles to improve conservation

measures in this population. One hundred and eighty-eight birds were studied: 181 chicks,, two juveniles, one subadult and four adults. The birds were externally examined and gross lesions at the oropharynx registered. Samples from the oropharyngeal cavity were obtained for *Trichomonas* spp. detection by culture and PCR, and positive samples were subjected to a multi-locus sequence typing approach, including the ITS1/5.8S/ITS2 region (ITS), ribosomal RNA small subunit (18S) and Fe-hydrogenase gene (FeHyd). Global prevalence for *T. gallinae* infection was 37.8% in total, 45.5% in nestlings. Thirty three percent of the birds developed lesions that ranged from mild (n=41) to moderate (n=14) or severe (n=7). MLST analysis showed five different MLS types, being ITS-A/18S-VI/FeHyd-A1 and ITS-D/18S-II/Fe-C4 the most frequent. An association between ITS-A/18S-VI/FeHyd-A1 and moderate or severe lesions was observed, but birds with type ITS-A/18S-VI/FeHyd-A2 also developed lesions. On the contrary, birds with MLS type ITS-D/18S-II/FeHyd-C4 displayed only a low proportion of mild lesions. Chicks raised in nests were at higher risk for *T. gallinae* infection and development of lesions than chicks raised in captivity. Disconrdances between samples cultured in TYM and samples subjected to PCR from oropharyngeal swabs were observed, being swab-ITS-PCR more sensitive.

KEY WORDS: Bonelli's eagle, nestlings, *T. gallinae*, MLST genotypes, gross lesions, ITS genotype, 18-S genotype, Fe hydrogenase genotype

Research highlights

45.5% of Bonelli´s eagle at nest carried *T. gallinae* and 39.4% showed lesions

PCR from oral swabs showed higher sensitivity than culture in TYM for detection of *T. gallinae*

MLS types ITS-A/18S-VI/Fe-A1 (& A2) are a risk factor for the development of lesions

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Introduction

Avian trichomonosis is a recognised emergent disease that affects wild bird species over the world (Lawson *et al*., 2006; Forzán *et al.*, 2010; Robinson *et al.*, 2010; Gourlay, 2011; Ganas *et al.*, 2013). The parasite is well known in columbiform species, the reservoir host, in which is found mainly in asymptomatic birds with a high prevalence rate (44.8% global prevalence in rock pigeons (*Columba livia*) with 0.37% of lesions in Sansano-Maestre *et al.*, 2009, and 79.4% of infection with no gross lesions in Martínez-Herrero *et al.*, 2014). Clinical symptoms are also reported from Columbiformes, with caseonecrotic granulomas that occupy the lumen of the alimentary canal, mostly at the oropharyngeal cavity, crop, and proximal oesophagus. Birds will be unable to swallow food due to the tissue inflammation and necrotic masses and may die by starvation (Forrester and Foster, 2008). Raptor species become infected by acquiring the protozoan by predation on preys that harbour the parasite or by direct contact through shared food or water. Indeed, avian trichomonosis was the main infectious disease of raptors in a long-term study from one of the Spanish wildlife recovery centres with a higher number of admissions (Molina-López *et al.*, 2012).

Bonelli's eagle (*Aquila fasciata*) is one of the most endangered raptor species in Europe. A drastically declining population is seen throughout its worldwide distribution range since the 1950's (BirdLife International 2020). Several factors are known as causes of this decrease: electrocution on power lines, direct persecution by shooting or poisoning and habitat modifications, which lead to a reduction of preferred prey species such as rabbits (*Oryctolagus cuniculus*) and red-legged partridges (*Alectoris rufa*),(Ferguson-Lees and Christie 2001; Real *et al*., 2001; Barov and Derhé 2011) and trichomonosis in nestlings (Höfle *et al*., 2000; Real *et al*., 2000). In other raptor species, like the Golden eagle (*Aquila chrysaetos*), a shift in the diet favouring the consumption of rock pigeons has been associated with the increase of trichomonosis in nestlings (Dudek *et al*., 2018).

The Iberian Peninsula harbours 80% of the western breeding European population of Bonelli’s eagles (Muñoz and Real, 2013). Different measures, including European Union LIFE programs (LIFE program NAT/ES/000701-Integral recovery of Bonelli's eagle population in Spain), have been implemented to assure the viability of the species in recent years. Avian trichomonosis is a

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disease of concern for this raptor species, due to the inclusion of pigeons on their diet range, especially when key species like rabbits and red-legged partridges are scarce (Palma *et al.*, 2006). The increase of predation on Columbiformes implies a major risk of death by avian trichomonosis and is a difficult conflict in some areas, mainly at the south-eastern Spain, where racing pigeons are part of the historical heritage of the region.

A high rate of infection in nestlings has been shown in previous studies, with 41% of the broods affected in Catalonia (Spain) and up to 87.5% with oropharyngeal lesions compatible with avian trichomonosis in the south-west of Portugal (Höfle *et al.*, 2000; Real *et al.*, 2000). Besides, a more recent study showed 52.4% of the eagles infected with *T. gallinae* (Sansano-Maestre *et al.*, 2009). Some of these reports also indicated that Columbiformes were a major part of the diet of the eagles. Thus, avian trichomonosis is one of the factors that can negatively contribute to the breeding success of the species and the real impact on the population trend has not fully been documented yet.

Additionally, different aetiologic agents of avian trichomonosis have been discovered recently: a novel *Trichomonas* sp. (Anderson *et al.*, 2009), *Simplicomonas* sp. (Ecco *et al.*, 2012), *Trichomonas stableri* (Girard *et al.*, 2014) and *Trichomonas gypaetinii* (Martínez-Díaz *et al.*, 2015). Consequently, a genetic characterization of clinical cases in wildlife species can offer unexpected insights.

The objective of this work was to determine the importance of avian trichomonosis in Bonelli's eagle during several avian trichomonosis screening campaigns carried out in nestlings from different geographic areas of Spain. In addition, the analysis was also applied to some birds admitted at wildlife recovery centres.

Material and methods

Birds examination and sampling.

Bonelli's eagle nestlings and chicks bred in captivity were examined as part of different conservation projects (LIFE program NAT/ES/000701-Integral recovery of Bonelli's eagle population in Spain; Marking and disease analysis program in Bonelli's eagle chicks from Comunitat Valenciana).

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Nestlings were sampled from 2014 to 2017 from the autonomous communities of Andalucía, Comunidad Valenciana, Castilla-La Mancha, Islas Baleares, and Madrid. A map showing the location of the sampled nest was constructed employing the free software gvSIG (Version: 2.3.1–2501, gvSIG Association) (Fig. 1).

Nestlings from 82 nests studied included chicks from 30 to 47 days of age, during the months of April and May (n=132). Birds were thoroughly examined externally by the veterinarians, including the oropharynx, and observed lesions compatible with oropharyngeal avian trichomonosis were classified as mild, moderate, or severe, according to criteria previously published by Martínez-Herrero *et al.* (2020). Oropharyngeal swab samples were taken from all the birds and official rings from the Ministry of Agriculture and Comunidad Valenciana were also placed by authorized scientists. The rest of the samples from chicks included birds bred in captivity in the facilities of GREFA (Majadahonda, Madrid) and associated centres in France: Center UFCS (LPO) in Vendée region, and center of Jean-Claude Mourgues in "Ardéche" region.

Cases with lesions compatible with trichomonosis were treated using carnidazole (20 mg/kg, PO, single dose). Moderate and severe cases were transferred to the recovery centre and treated by surgery when the extension of the granulomas occupied more than 50% of the oropharyngeal tissues or crop.

In addition, some adults and juveniles were sampled after admission at different recovery centres (“La Granja de El Saler” from Valencia province; “Forn del Vidre” from Castellón province; “El Blanqueo” from Granada province).

Sources of samples.

Samples for the study of *Trichomonas* spp*.* were taken with a sterile cotton swab from the oropharyngeal cavity and crop. Swabs were moistened in culture medium prior to use. Tubes with 5 ml of liquid Tryptose-Yeast-Maltose (TYM) medium, supplemented with 10% foetal bovine serum and antibiotics, were inoculated and incubated at 37ºC for 10 days. Cultures were daily monitored in an inverted microscope. The obtained isolates were cryopreserved at -80ºC with 5% dimethyl sulfoxide (DMSO) in TYM medium for further studies. An additional oropharyngeal swab was taken from some birds in 2015 (n=37/52 chicks) and 2016 (n=38/58

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chicks), and from all birds in 2017, frozen at -20ºC and subjected to DNA extraction for the genetic characterization of the parasite.

DNA isolation and PCRs.

DNA was extracted from 1 ml of positive cultures at passage 0, or from oropharyngeal swabs using a commercial kit (DNeasy Blood and Tissue Extraction Kit, QIAGEN, Valencia, CA, USA). A multilocus sequence typing (MLST) analysis was performed including the ITS1/5.8S/ITS2 region (ITS), a fragment of the Fe-hydrogenase gene (FeHyd) and ribosomal RNA small subunit (18S) gene. Oligonucleotide primers were: TFR1 (5'-TGCTTCAGTTCAGCGGGTCTTCC-3') and TFR2 (5'-CGGTAGGTGAACCTGCCGTTGG-3') for the ITS, Hm-Long-f (5'-AGGAAGCACACTATGGTCATAG-3') and Hm-Long-r (5'-CGTTACCTTGTTACGACTTCTCCTT-3') for the 18S, and Fe-hyd-for (5'-GTTTGGGATGGCCTCAGAAT-3') and Fe-hyd-rev (5'-AGCCGAAGATGTTGTCGAAT-3') for the FeHyd.

Temperature profiles were used according to Ganas *et al.* (2013). For both the ITS and 18S: 15 min at 95º C for initial denaturation, 40 cycles of 30 s at 94ºC, 1 min at 55ºC, 2 min at 72ºC and a final extension of 10 min at 72ºC. In the case of FeHyd: 15 min at 95º C for initial denaturation, 40 cycles of 30 s at 94ºC, 1 min at 58ºC, 2 min at 72ºC and finally, 10 min at 72ºC. All reactions were carried out in a GeneAmp 2700 thermal cycler (Applied Biosystems, Foster City, California, USA). Amplified products were analysed by electrophoresis in 1% agarose gels stained with ethidium bromide. Results were observed under UV light in a transilluminator.

PCR of the ITS1/5.8S/ITS2 region (ITS) was applied to the DNA extracted from the swab (swab-ITS-PCR) to detect *Trichomonas* spp. infection.

Sequence analysis.

Amplicons were submitted to the laboratories of Sistemas Genómicos, S. A. (Paterna, Valencia, Spain) for Sanger sequencing in a 3730XL DNA Analyzer with the ABI PRISM® BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA). Sequence chromatograms were examined in both directions and assembled with Lasergene SeqMan software version 7.0.0 (DNASTAR, Madison, WI, USA). Nucleotide Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi/) version 2.3.1 was employed for the

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genetic study, using the Mega BLAST algorithm (optimization for highly similar sequences) and low complexity region filter. A homology of 100% and full coverage were observed in all sequences when compared with others at GenBank database (http://www.ncbi.nlm.nih.gov/genbank/).

Phylogenetic analysis.

Three phylogenetic trees were constructed employed sequences available at Genbank and previously published by other authors. Ten Sequences of the ITS region and with a minimum length of 290 nucleotides were included, and a sequence of *T. foetus* was included as an out-group reference. Twelve sequences of the 18 S region and with a minimum length of 740 nucleotides were included, and a sequence of *Pentatrichomonas hominis* was included as an out-group reference. Twenty three sequences of the FeHyd region and with a minimum length of 521 nucleotides were employed, including sequences of *T. vaginalis* and *T. stableri*. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1983). The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018). Bootstrap of 2000 replicates was employed to estimate trees reliability.

Analysis of risk factors.

A Chi square test was used to evaluate differences in infection rates between nest-raised and captive-raised birds.. Risk factors were evaluated using Odds Ratio determination at 95% confidence interval. A risk factor analysis was performed, employing Odds ratio at 95% confidence interval analysis, to evaluate the relationship between MLS type and the presence of moderate and severe lesions in the birds studied. The relationship between nest or captive rearing on the development of lesions was also analyzed. Free software WinEpi (http://www.winepi.net/) was employed for the statistical analysis.

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Concordance between the diagnostic methods employed to detect the parasite, culture in TYM and PCR of the ITS fragment from oropharyngeal swabs (swab-ITS-PCR), was calculated using *kappa* statistic at 95% confidence interval. The evaluation of the diagnostic approaches was determined by the values of sensitivity, specificity, positive and negative predictive values, Jouden´s J statistic and reliability at 95% confidence.

Results

Samples of birds and *T. gallinae* infection.

One hundred and eighty-eight birds were sampled, being 96.3% chicks (n=181). One hundred and thirty-two nestlings were examined along the sampling period in 82 nests, most of them with two brothers: eight in 2014, 23 in 2015, 26 in 2016 and 25 in 2017. Most of the studied nests (n=50) harboured two birds, ranging the productivity (nestlings per monitored nest) from 1.56 to 1.69. Nests with three birds were not found. The rest of the chicks (n=48) were bred in captivity in two centres from France (n=36) and at the facilities of GREFA (Majadahonda, Madrid) (n=12). Four adults, one subadult, two juveniles and one chick admitted at different recovery centres in 2014 and 2015 were also included in the study (supplementary tables 1 and 2).

Trichomonas infection was detected in 37.8% (n= 71/188) of the birds, 45.5% (n=60/132) of the wild nestlings and 16.7% (8/48) of the chicks raised in captivity, considering those with a positive culture or a positive swab-ITS-PCR. In those cultures with scarce growth (n=12), mainly due to bacterial or fungal overgrowth or to a long time between sampling and incubation, DNA was also extracted and a PCR of the ITS fragment was carried out. Only three birds from the eight admitted at wildlife recovery centres were positive to *Trichomonas* infection.

Positive rate of *T. gallinae* infection in chicks from nests varied among the sampled years: in 2014, 30.8% positive animals (n=4/13) and 37.5% of nests infected (n=3/8) were detected, while in 2015 positive rate was 52.8% (n=19/36) and 65.2% of nests were infected, (n=15/23). In 2016, 40.9% (n=18/44) of chicks were positive to the parasite and 61.5% (n=16/26) of nests were infected and in 2017, 50% of birds (n=20/40) and 64% of nests were infected (n=16/25).

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During the study period, some nests were sampled in more than one breeding season (49 chicks, 14 nests). Some of the nests were systematically infected with the parasite (n=3), while others were found always negative (n=3). Three nests were negative at the first sampling year and positive the following years. Five nests were positive during the first sampling occasion and then negative the next year. Two nests had a chick with a moderate lesion compatible with *T. gallinae* only the first time they were sampled, but no infection could be confirmed, since PCR from the swab was not performed at that time to all the birds. Severe lesions were found in two chicks from different nests: one of them came from a nest systematically infected with the parasite, and ITS A genotype was always present. The other chick came from a nest where the broods showed lesions compatible with *T. gallinae* infection in a two year-round, but the first year the parasite was not detected (again, no swab-ITS-PCR was done), while the second year, ITS A genotype was found.

Agreement between culture and swab-ITS-PCR for detection of *Trichomonas*.

One hundred and twenty-six samples were tested by both diagnostic techniques, swab-ITS-PCR and culture in TYM medium (supplementary table 3). In total, 52 samples were positive to one or both techniques, but swab-ITS-PCR detected more positive samples (n=43) than culture in TYM (n=19). Considering infected birds those with positive results to one or both techniques, sensitivity and predictive values were calculated. Specificity was considered as 100% since in all the samples positive to the culture, a movement compatible with *Trichomonas* spp. was observed and sequences from cultures or swabs showed 100% homology with *T. gallinae*, and thus, no false positive results were obtained. Swab-ITS-PCR displayed a sensitivity of 82.7% (95% CI=72.4, 93), same value as Youden´s J statistic, a positive predictive value of 100% (same value than specificity) and a negative predictive value of 89.2% (95% CI= 82.5, 95.8). Reliability was 92.9% (95% CI=88.4, 97.3). Only nine birds were detected by culture in TYM but not by swab-ITS-PCR.

Culture in TYM displayed a much lower sensitivity, 36.5% (95%CI=23.5, 49.6). Negative predictive value was 69.2% (95%CI=60.4, 77.9). Reliability of culture in TYM was 73.8% (95% CI=66.1, 81.5).

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Concordance between both techniques was scarce (*kappa* value=0.143, 95% CI=0.023, 0.264). Only 10 samples were positive to both techniques, while 74 were negative to both diagnostic tests and 42 samples showed disagreement between them, with one being positive and the other negative.

Birds lesions.

Gross lesions were found in 33% (n=62) of the birds, of which 59.7% (n=37) were positive to *T. gallinae* by culture or swab-ITS-PCR and 40.3% (n=25) were negative to the parasite (supplementary table 2). Among the birds without macroscopical lesions (n=126), 27% were positive to the parasite (n=34). Macroscopical lesions compatible with avian trichomonosis, from the 62 chicks, ranged from mild (n=41, 33 from nests and eight from captivity breeding) to moderate (n=14 from nests) or severe lesions, including caseonecrotic granulomas that occluded the alimentary tract (n=7, five from nests and one chick and one adult from admissions at wildlife recovery centres) (Figure 2).

Some birds with mild lesions were negative to *T. gallinae* (34.1%, n=14/41). From birds with moderate lesions, 71.4% were negative to the parasite (10/14), although they were sampled at 2014 and 2015, when swab-ITS-PCR was applied to less than half of the samples (6/14), from which, three were positive and three negative to the PCR. All the birds with severe lesions were positive to *T. gallinae*, with one exception (n=6/7), but only one of them was positive by culture.

Multilocus sequence typing Analysis.

Genotyping was possible in 71 infected birds (Table 1). In 33 samples, amplification and sequencing of the three targets was successfully determined. ITS genotype could be obtained from all positive samples, while 18S sequences were obtained only from 52 samples (73.2% of positive samples) and FeHyd genotype was determined in only 33 samples (46.5% of positive samples), this one being the target with the lowest detection capacity. Mixed infections were also found in 10 birds (n=14.1%). Phylogenetic trees including sequences found in this study are shown in figure 3.

Three genotypes of the ITS region (nomenclature according to Gerhold *et al*., 2008) were detected, being A and D predominant. Genotype A (EU881911 of Sansano-Maestre *et al*.,

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2009; genotype A of Gerhold *et al*., 2008; genotype IV of Grabensteiner *et al*., 2010 and genetic group ITS-OBT-Tg-1 of Martínez-Herrero *et al.*, 2014) in 28 birds and genotype D (EU881912 of Sansano-Maestre *et al*., 2009; genotype D of Gerhold *et al*., 2008; genotype I of Grabensteiner *et al*., 2010 and genetic group ITS-OBT-Tg-2 of Martínez-Herrero *et al.*, 2014) in 33 birds. Ten birds showed mixed sequences compatible with both genotypes, according to the sequence chromatograms. The third genotype was found only in one animal (16-0699) and showed 100% homology with a sequence isolated from a racing pigeon (i.e. FN433473, genotype III of Grabensteiner *et al*., 2010).

Analysis of the 18S region revealed the presence of three genotypes, although two of them predominated over all. Genotype VI (FN433484, Grabensteiner *et al.,* 2010) was found in 28 birds and genotype II (FN433480, Grabensteiner *et al.,* 2010) was found in 21 birds.. Finally, one sequence showed 100% homology with several sequences, among them, sequence MK932773, isolated from a European turtle dove (*Streptopelia turtur*) by Santos *et al.* (2019) and sequence FN433486, isolated from a racing pigeon (Genotype VIII, of Grabensteiner et al., 2010). Two birds displayed mixed sequences compatible with genotypes VI and II, according to the chromatograms.

Fe-Hyd gene revealed that three genotypes were present, but once again, two of them predominated over the others. Genotype A1 (HG008114, Ganas *et al*., 2014) was found in 18 birds while genotype C4 (KC529662, Chi *et al*, 2013) was found in 10 birds. Genotype A2 (HG008115, Ganas *et al*., 2014) was found in three birds. Two birds displayed mixed infections compatible with the predominant genotypes A1 and C4, according to the chromatograms.

MLST analysis demonstrated that Bonelli's eagles were infected with different *T. gallinae* strains. Five MLS types were clearly determined in 28 birds, with the three targets sequenced excluding mixed sequences. Two MLS types predominated over all: multilocus sequence (MLS) types ITS-A/18S-VI/FeHyd-A1 (n=10) and ITS-D/18S-II/FeHyd-C4 (n=14). MLS type ITS-A/18S-VI/FeHyd-A2 was found in three birds and MLS type ITS-D/18S-II/FeHyd-A1 was displayed by one animal. Most samples showed agreement between the genotypes of the three targets sequenced, but discordances were seen in three samples (one ITS-D/18S-II/FeHyd-A1 and two ITS-D/18S-VI/FeHyd unsuccessful).

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In many cases, positive PCR and sequences were obtained from one of the nestlings of each nest, but not from the brother. Only in 13 of the 82 sampled nests, sequences from two nestlings were obtained. In eight nests agreement between genotypes from both brothers was obtained, but in other three nestlings mixed sequences were obtained and in two nests a disagreement was observed, although some of the targets were not successfully sequenced.

Analysis of risk factors: breeding and genotype.

A high percentage of nests was found positive to the parasite (58.5%, n=48/82). Birds raised in captivity showed significantly lower level of infection (16.7%, n=8/48) than those raised in nests (45.5%, 60/132) (p=0.0004). Thus, birds bred in nest were at higher risk of infection by *T. gallinae* (OR=4.17, 95% CI 1.81-9.58).

Chicks raised at nest showed lesions more frequently than chicks raised in captivity (52/132 *vs.* 8/48), and nest was considered as a risk factor to develop lesions in comparison with captivity raising (OR=3.25, 95% CI 1.41-7.49). Birds raised in captivity developed only mild lesions, while birds bred in nest developed mild (n=33/52), moderate (n=14/52) and severe lesions (n=5/52).

Eleven out of fourteen birds (78.6%) with MLS type ITS-A/18S-VI/FeHyd-A1 developed lesions: seven mild, one moderate and three severe, while three out of 10 birds (33.3%) of type ITS-D/18S-II/FeHyd-C4 developed solely mild lesions. Then, birds with MLS type ITS-A/18S-VI/FeHyd-A1 were at higher risk to develop lesions than birds of type ITS-D/18S-II/FeHyd-C4 (OR=8.55, 95% CI 1.33-54.95).

For those birds with incomplete MLS types of the three targets, only some birds harbouring ITS A genotype developed moderate or severe lesions. On the contrary, a small proportion of birds with ITS D genotype developed mild lesions, but none of them developed moderate or severe lesions. Moreover, all the birds displaying moderate and severe lesions harboured A-ITS genotype and 18S genotype VI and, in birds with Fe-Hyd region sequences, genotypes A1 and A2 were present, except for one animal with mixed sequence at ITS.

Discussion

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Trichomonosis is still a menace to Bonelli´s eagle chicks, but the positive rates obtained in different studies varies from 6.3% to 68.8% (Höfle *et al*. 2000; Real *et al*, 2000; Sansano-Maestre *et al*., 2009; Santos *et al*., 2019), perhaps due to differences between years, the age of the chicks at sampling, different sampling areas or diagnostic techniques employed. Positive rate could also vary depending on the prey items of the Bonelli´s eagles, increasing when Columbiformes constitute a high percentage of the diet (Real *et al*., 2000; Palma *et al*., 2006; Resano-Mayor *et al*., 2014). According to Real *et al.* (2000), the age of the parents is another factor that could influence the infection rate of the parasite, being parents less than four years old more prone to developing the infection.

In the present study, 45.5% (n=60/132) of the Bonelli´s eagle wild nestlings were infected with the parasite, but only 28% were positive by culture (n=37/132), which agrees with data from Real *et al.* (2000), who found 36% of nestlings infected. However, positive rate increased from 23.1%, when only culture was used (year 2014, n=3/13), up to 40.9-52.8% when swab-ITS-PCR was included within the diagnostic techniques (n=19/36 in 2015, n=18/44 in 2016 and n=20/39 in 2017), since some samples were positive only by swab-ITS-PCR. The absence of concordance between culture in TYM and swab-PCR obtained in the present study points out to one of the reasons related to the differences found between studies. Maybe some isolates do not grow properly in the culture media, or perhaps the time between sampling and incubation was too long. Among the studies, Santos *et al.* (2019) obtained the lowest positive rate (6.3%) in Bonelli´s eagle nestlings of the Iberian Peninsula. According to the authors, the small number of samples or a long-time interval between sampling collection and incubation could be an explanation for the low values, even if lesions compatible with trichomonosis were present. The same happened to us when nests were located far from the laboratory, which means more than 24 hours from sampling to the incubator, and for this reason, we introduced the swab-ITS-PCR to detect the parasite. In total, 24/62 (36.9%) birds negative to culture and sampled far from the laboratory were positive to ITS-PCR (from culture sediment or swab), while 5/42 (11.9%) of the samples negative to culture and sampled near the laboratory (less than 8 hours) were positive to the ITS-PCR.

Only 33% of the sampled birds showed lesions at the oropharynx, most of them being mild lesions. Of these, 59.7% were positive to *T. gallinae*. Mild lesions can be due to other causes or

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even to minor injuries, which could explain the low detection. However, moderate and especially severe lesions at the oropharynx, considered as pathognomonic of trichomonosis were found in 10.6% (n=14/132) and 3.8% (n=5/132), respectively, of the nestlings. Our data agrees with observations made by Real *et al.* (2000), who found between up to 6% of nestlings with severe lesions (> 2 cm nodule size) during eight years of study. Other authors found higher percentages of gross lesions, especially in Portugal, and the percentage of severe cases were slightly higher: Höfle *et al* (2000) found 32% of nestlings with gross lesions and 10.1% mortality in nestlings attributed to the disease, and Santos *et al.* (2019) reported 12.5% of nestlings with gross lesions of trichomonosis.

When birds with moderate lesions were examined for *T. gallinae*, 71.4% were negative to the parasite (10/14), although in 2014 swab-ITS-PCR was not implemented and in 2015 the technique was applied to four out of seven samples, from which, three were negative and one positive to the PCR. Two of the birds with moderate lesions belonged to a nest infected with *T. gallinae*, since their brothers harboured the parasite.

Only one positive culture was obtained from the birds with severe lesions, another animal was positive at the PCR of the culture sediment, and other four samples were positive solely at swab-ITS-PCR, while the animal negative to *T. gallinae* shared nest with a chick positive to the infection. Santos *et al.* (2019) and Höfle *et al.* (2000) also found it difficult to obtain positive cultures from birds with gross lesions, but this problem has been partly resolved with the present approach and *T. gallinae* was confirmed in most of the samples with pathognomonic lesions.

MLST analysis of the present study was carried out using three targets: ITS region, 18S region and FeHyd region. ITS PCR was the most sensitive (n=71), followed by 18S PCR (n=52, 73.2% of positive samples) and then by FeHyd PCR (n=33, 46.5%). These results were expected, since FeHyd is a single copy gene, while the other two regions are multi-copy regions.

In general, when all the targets were successfully sequenced, an agreement between genotypes was observed: birds with genotype ITS A showed genotype VI at 18S and genotypes A1 or A2 at FeHyd, while birds with genotype ITS D showed genotype II at 18S and genotype C4 at FeHyd. Only one bird showed genotype ITS III and 18S VIII, according to Grabensteiner

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et al., (2010). Also, one animal displayed genotype FeHyd A1 and genotype ITS D. Besides, 12 birds displayed mixed sequences at one or more targets. These results are in agreement with those obtained by Grabensteiner *et al.* (2010), who found birds harbouring infections with more than one genotype. Also, Alrefaei *et al.* (2019a, b) suggests that gene flow between trichomonads, even among distant trichomonads is possible, and hybrid trichomonads genomes could emerge.

Two of the five MLS types described here were widely distributed, and they represented 85.7% of the complete sequenced samples: ITS-A/18S-VI/FeHyd-A1 (n=10/28) and ITS-D/18S-II/FeHyd-C4 (n=14/28). Both types were present in all the regions analysed in Spain and no predominance in any territory was observed. Comparisons with other studies carried out in Bonelli´s eagles are not easy, because the three targets were not included in most studies. Santos *et al.* (2019) described type 18S II /ITS D in one eagle without lesions, which could coincide with ITS-D/18S-II/FeHyd-C4, although FeHyd was not included in their study. The most frequent MLS type from the area studied by the authors included genotype B of Gerhold and VI 18S, which could coincide in the targets sequenced with our ITS-A/18S-VI/FeHyd-A1. Genotypes A and B of Gerhold *et al.* (2008) were more than 99% identical, and we previously referred to them as ITS-OBT-Tg-1 (Martínez-Herrero *et al.,* 2014). Quillfeldt *et al.* (2018) also grouped these two types because they are almost identical and frequently associated with lesions (they reported A/B ITS types in different species of Columbiformes, raptors and finches with gross lesions). Sansano-Maestre *et al.* (2016) genotyped ITS and FeHyd and found ITS A/B and FeHyd A1 in two samples of Bonelli´s eagles, which coincides with our MLS type ITS-A/18S-VI/FeHyd-A1 without the 18S sequence. In the same study, ITS-D/FeHyd-C4 and C5 were reported, displaying a similar proportion of ITS genotypes reported in the present study.

In other parts of the world, where MLS typing was applied to other bird species, MLS types ITS-A/18S-VI/FeHyd-A1 and ITS-D/18S-II/FeHyd-C4 have been found widely distributed, with fine-scale variations. Both were reported in Columbiformes and other non-passerines from UK (Chi *et al*., 2013), birds of prey and prey birds from Spain (Martínez-Herrero *et al*., 2014), finches and pigeons from Canada (McBurney *et al*., 2015), finches from Fennoscandia (Lawson *et al*., 2011) and Columbiformes from Portugal (Santos *et al*., 2009). In some of the previous studies, however, the typing was done with only two targets.

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Finally, but not less important, a clear relationship has been observed between MLS type ITS-A/18S-VI/FeHyd-A1 and the presence of moderate and severe lesions. The hypothesis of increased virulence of genotype ITS A was put forward by Sansano-Maestre *et al.* in 2009, and later confirmed by our group and several other authors: Lawson *et al* (2011a), Chi *et al* (2013), Martínez-Herrero *et al.* (2014), McBurney *et al.* (2015), Sansano-Maestre *et al*. (2016). Most of these studies included at least two targets, ITS A/B and FeHyd A1 and A2 were the genotypes present in most of the cases with gross lesions. Moreover, the epidemic finch strain from the UK (ITS A, FeHyd A1, Lawson *et al*., 2011a) was the most frequent of the types found in birds with macroscopic lesions in all epidemiological studies. These observations are in agreement with the *in vitro* assays of virulence on cell cultures of ITS-A/18S-VI/FeHyd-A1 type clones and the differences in membrane protein composition (Martínez-Herrero *et al*., 2019). In the above-mentioned study, three clones of ITSA/18S-VI/FeHyd-A1 coming from birds with lesions were used, although in two of them 18S sequences were 100% homologous to KM095107, which is slightly longer than 18S-VI from Grabensteiner *et al.* (2010), but identical in nucleotide composition in the common sequence. Then, the same MLS type was determined in all the employed clones. The clones were obtained from different bird species (*Bubo bubo, Columba palumbus* and *Falco tinnunculus*) and in different sampling years (2012 and 2013). Results pointed out to a reorganization of the cytoskeleton related to the virulence, although the analysis of more isolates would be desirable (Martínez-Herrero *et al*., 2019). In this study, we have demonstrated a clear association between type ITS-A/18S-VI/FeHyd-A1&A2 and development of gross lesions using only one bird species, excluding some of the possible confounders, like bird species, in the risk analysis.

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Disclosure statement

The authors declare that there is no competing interests on their research.

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Figure 1. Map showing the sampled areas. Each dot represents one location with one or more nests studied.

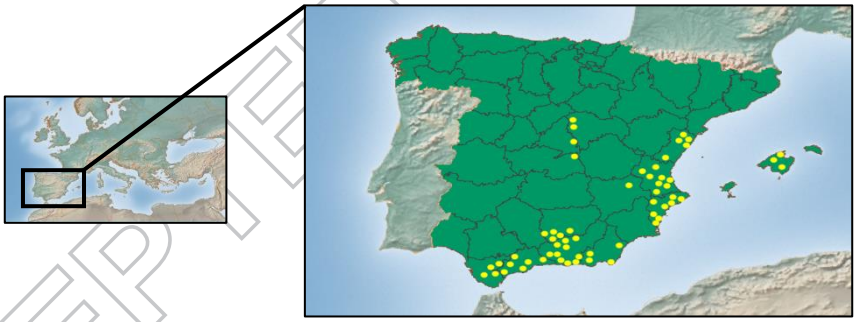

Figure 2. Lesions found in Bonelli´s eagles from this study. A: mild lesion. B: moderate lesion. C: severe lesion.

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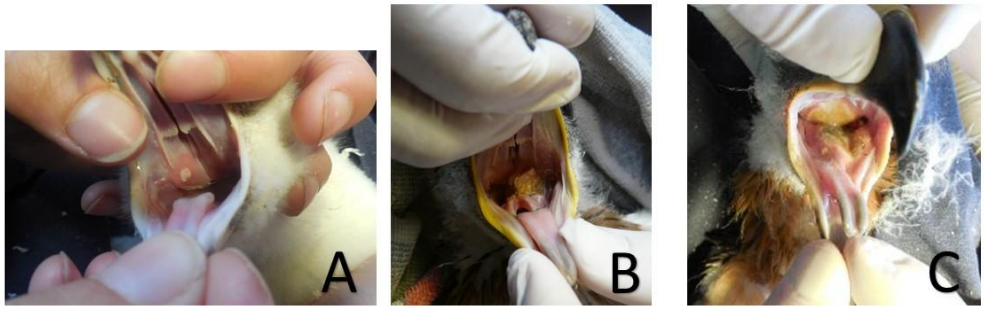
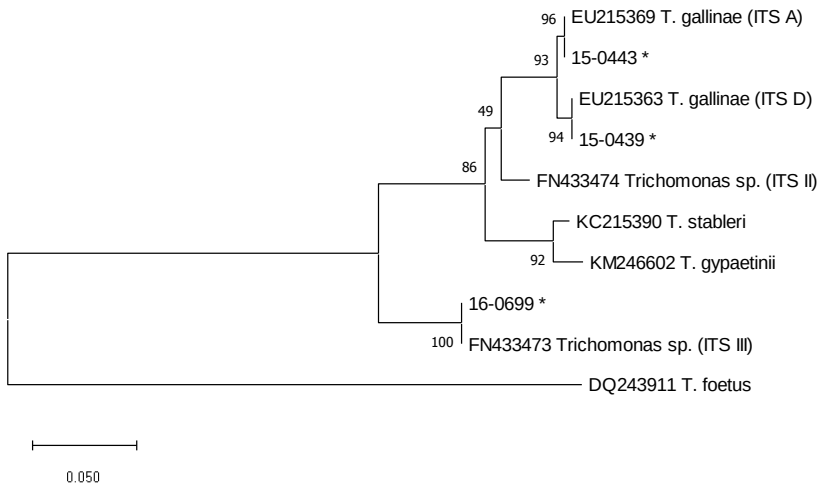


Figure 3. Phylogenetic trees of ITS (A), 18S (B) and FeHyd (C) genes including sequences previously reported by other authors, and sequences obtained in this study (*). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value (Tamura and Nei, 1993). The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018). *: A representative sequence of each genotype found in this study is included in each phylogenetic tree. ITS genotypes according to Gerhold *et al*. (2008) and Grabensteiner *et al*. (2010). 18S genotypes according to Grabensteiner *et al*. (2010). FeHyd genotypes according to Lawson *et al*. (2011b), Ganas et al. (2014) and Alrefaei *et al*. (2019a).

A



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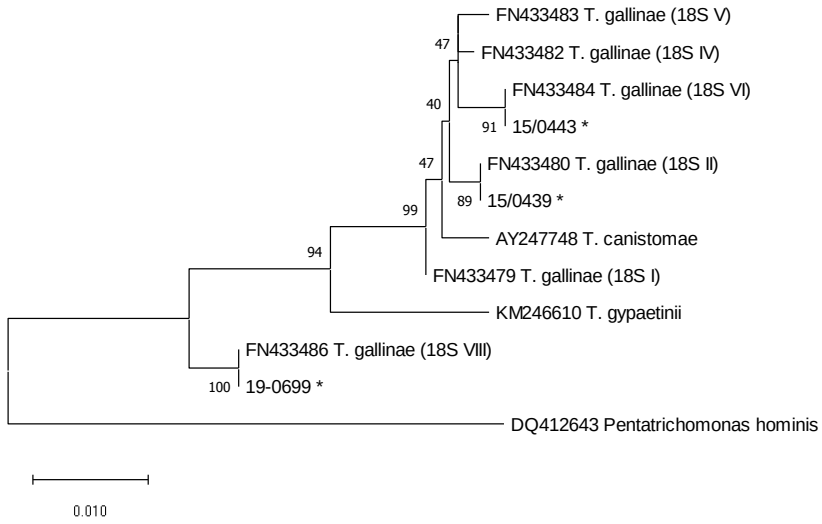
B



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C

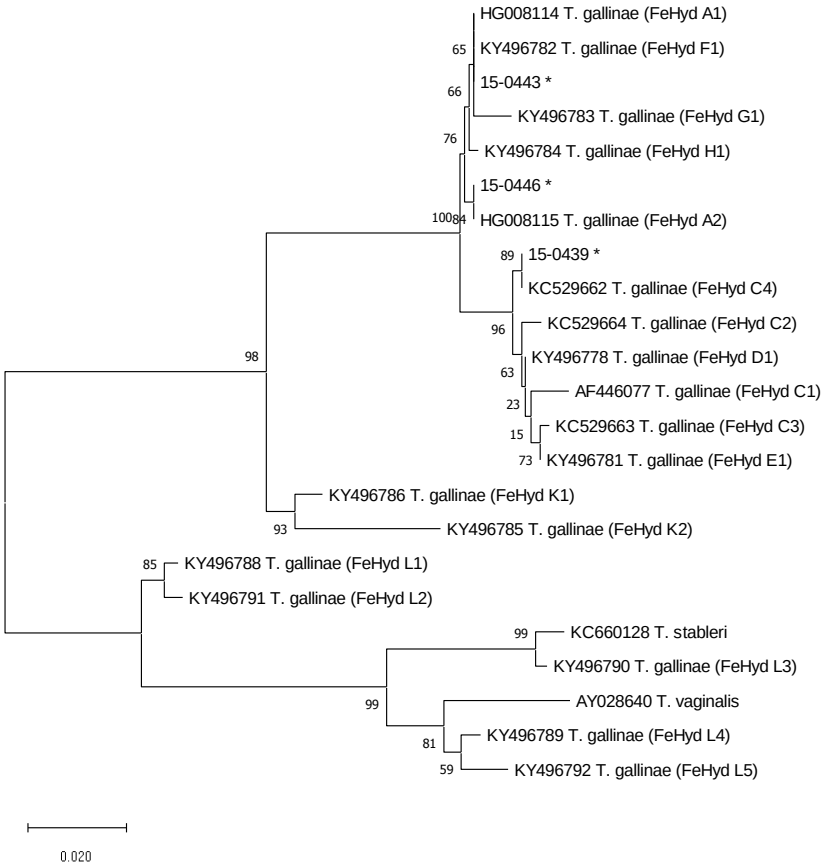

Table 1. MLST results of oropharyngeal trichomonads from Bonelli's eagle (*Aquila fasciata*) and number of birds without lesions or with mild, moderate or severe lesions. n: number of samples. The analysis involved the ITS1/5.8S rRNA/ITS2 (ITS), the small subunit of rRNA (18S) and Fe-hydrogenase (FeHyd) gene sequences.

MLS type[a, b, c] (n)	**ITS[a]**	**18-S[b]**	**FeHyd[c]**	**No Lesion**	**Mild Lesion**	**Moderate Lesion**	**Severe Lesion**

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A-VI-A1 (14)	A	VI	A1	3	7	1	3
A-VI-A2 (3)	A	VI	A2	0	1	0	2
A-VI (6)	A	VI	-	2	2	2	1
A-A1 (1)	A	-	A1	0	1	0	0
A- (3)	A	-	-	0	3	0	0
D-II-C4 (10)	D	II	C4	7	3	0	0
D-II-A1 (1)	D	II	A1	1	0	0	0
D-II (8)	D	II	-	7	1	0	0
D-VI (2)	D	VI	-	1	1	0	0
D- (11)	D	-	-	9	2	0	0
IIIG-VIII (1)	D	VIII	-	1	0	0	0
A/D-VI-A1 (2)	MIXED (A - D)	VI	A1	0	2	0	0
A/D-II (2)	MIXED (A - D)	II	-	0	1	1	0
A/D- (4)	MIXED (A - D)	-	-	2	2	0	0
A/D-VI/II-A1/C4 (2)	MIXED (A - D)	MIXED (VI-II)	MIXED (A1-C4)	1	1	0	0
Total 71				34	27	4	6

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[a]: ITS genotype nomenclature according to Gerhold *et al.* (2008) and comparison of Sansano-Maestre *et al.* (2009) and Grabensteiner *et al.* (2010). A: EU881911 (Sansano-Maestre *et al* 2009, equivalent to genotype A of Gerhold *et al*., 2008 and genotype IV of Grabensteiner *et al*., 2010); D: EU881912 (Sansano-Maestre *et al*., 2009, equivalent to genotype D of Gerhold *et al*., 2008, and genotype I of Grabensteiner *et al*., 2010); IIIG (genotype III of Grabensteiner *et al*., 2010). [b]: 18S genotype nomenclature according to genetic groups of Grabensteiner *et al*., (2010). VI: FN433484; II: FN433480; I: FN433479. [c]: FeHyd genotypes nomenclature according to Chi *et al.* (2013) or with GenBank acc. n. A1: HG008114; A2: HG008115; C4: KC529662.

YEAR	SAMPLEID	LESION	LESIONGRADE	AGE	NEST	ORIGIN (Center, Province, Country)	NEST/CAPTIVITY/ADMISSION	TRICHOMONAS (Positive/Negative to Culture or PCR from Swab or PCR from Culture sediment)	GENOTYPE ITS *Trichomonas*	GENOTYPE 18S *Trichomonas*	GENOTYPE Fe *Trichomonas*	CULTURE	ITS PCR from CULTURE	ITS PCR from SWAB	CULTURE (GROWTH ORSEDIMENT PCR)
2017	337	NO	0	CHICK	2017-2	Castelló n	NEST	NEGATIVE				NEGATIV	Nd	NEGATIV	NEGATIV

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					2							E		E	E
2017	338	NO	0	CHICK	2017-22	Castelló n	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2017	351	NO	0	CHICK	2017-20	Valencia	NEST	NEGATIVE				NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
2017	352	NO	0	CHICK	2017-20	Valencia	NEST	NEGATIVE				NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
2017	353	NO	0	CHICK	2017-24	Castelló n	NEST	POSITIVE	ITS-D	II	-	POSITIVE	POSITIVE	NEGATIVE	POSITIVE
2017	354	NO	0	CHICK	2017-23	Castelló n	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2017	355	NO	0	CHICK	2017-23	Castelló n	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2017	356	NO	0	CHICK	2017-24	Castelló n	NEST	POSITIVE	ITS-D	II	C4	POSITIVE	POSITIVE	NEGATIVE	POSITIVE
2	3	N	0	C	2	Castelló	NES	NEG				N	N	N	N

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0 1 7	5 7	O		HI C K	0 1 7 - 2 1	n	T	ATI VE				E G A TI V E	d	E G A TI V E	E G A TI V E
2 0 1 7	3 6 4	N O	0	C HI C K	2 0 1 7 - 1 9	Valencia	NES T	NEG ATI VE				N E G A TI V E	N E G A TI V E	N E G A TI V E	N E G A TI V E
2 0 1 7	3 6 5	N O	0	C HI C K	2 0 1 7 - 1 9	Valencia	NES T	NEG ATI VE				N E G A TI V E	N d	N E G A TI V E	N E G A TI V E
2 0 1 7	3 6 6	Y E S	II	C HI C K	2 0 1 7 - 1 8	Valencia	NES T	POS ITIV E	ITS-A	VI	-	P O SI TI V E	P O SI TI V E	P O SI TI V E	P O SI TI V E
2 0 1 7	3 6 7	Y E S	II	C HI C K	2 0 1 7 - 1 7	Valencia	NES T	POS ITIV E	ITS-A	VI	-	P O SI TI V E	P O SI TI V E	P O SI TI V E	P O SI TI V E
2 0 1 7	3 6 8	N O	0	C HI C K	2 0 1 7 - 1 6	Valencia	NES T	NEG ATI VE				N E G A TI V E	N d	N E G A TI V E	N E G A TI V E
2 0 1 7	3 6 9	N O	0	C HI C K	2 0 1 7 - 1 6	Valencia	NES T	NEG ATI VE				N E G A TI V E	N E G A TI V E	N E G A TI V E	N E G A TI V E
2 0 1	1 7/ 0	N O	0	C HI C		GREFA	CA PTI VIT	NEG ATI VE				N E G	N d	N E G	N E G

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7	285			K			Y					ATIVE		ATIVE	ATIVE
2017	17/02866	NO	0	CHICK		GREFA	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2017	17/05311	YES	I	CHICK	2017-1	Granada	NEST	POSITIVE	ITS-A	VI	A1	POSITIVE	POSITIVE	POSITIVE	POSITIVE
2017	17/05322	YES	III	CHICK	2017-1	Granada	NEST	POSITIVE	ITS-A	VI	A1	NEGATIVE	Nd	POSITIVE	NEGATIVE
2017	17/05366	NO	0	CHICK	2017-2	Jaén	NEST	NEGATIVE				NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
2017	17/05377	NO	0	CHICK	2017-2	Jaén	NEST	NEGATIVE				NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
2017	17/06107	YES	I	CHICK		GREFA	CAPTIVITY	POSITIVE	MIXED SEQUENCE (ITS-A + ITS-D)	-	-	NEGATIVE	Nd	POSITIVE	NEGATIVE
2017	17/06577	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIV	NEGATIV	NEGATIV	NEGATIV

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2017	17/06/58	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	POSITIVE	ITS-D	-	-	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE
2017	17/06/59	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2017	17/07/13	NO	0	CHICK	2017-3	Granada	NEST	POSITIVE	ITS-D	II	-	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE
2017	17/07/19	NO	0	CHICK		Jean Claude Morgues, Ardéche (France)	CAPTIVITY	NEGATIVE				NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
2017	17/07/59	NO	0	CHICK	2017-4	Mallorca	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2017	17/08/93	YES	I	CHICK	2017-6	Cádiz	NEST	POSITIVE	ITS-D	VI	-	NEGATIVE	Nd	POSITIVE	NEGATIVE
2017	17/10/59	YES	I	CHICK	2017-5	Cádiz	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2	1	N	0	C	2	Jaén	NES	NEG				N	N	N	N

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017	7/10600	O		HICK	017-10		T	ATIVE				EGATIVE	d	EGATIVE	EGATIVE
2017	17/10611	NO	0	CHICK	2017-10	Jaén	NEST	POSITIVE	ITS-D	II	C4	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE
2017	17/10622	NO	0	CHICK	2017-9	Mallorca	NEST	POSITIVE	ITS-A	VI	A1	NEGATIVE	POSITIVE	POSITIVE	POSITIVE
2017	17/10633	YES	I	CHICK	2017-9	Mallorca	NEST	POSITIVE	MIXED SEQUENCE (ITS-A + ITS-D)	VI	A1	POSITIVE	POSITIVE	NEGATIVE	POSITIVE
2017	17/10666	NO	0	CHICK	2017-7	Cádiz	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2017	17/10677	NO	0	CHICK	2017-7	Cádiz	NEST	POSITIVE	ITS-D	II	-	NEGATIVE	Nd	POSITIVE	NEGATIVE
2017	17/10688	YES	I	CHICK	2017-8	Guadalajara	NEST	POSITIVE	MIXED SEQUENCE (ITS-A + ITS-D)	-	-	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE
201	17/1	NO	0	CHIC		UFCS Vendée (France)	CAPTIVIT	POSITIVE	ITS-D	-	-	NEG	Nd	POSI	NEG

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7	158			K			Y					ATIVE		TIVE	ATIVE
2017	17/11159	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	POSITIVE	ITS-D	-	-	NEGATIVE	POSITIVE	POSITIVE	POSITIVE
2017	17/11160	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	POSITIVE	ITS-D	-	-	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE
2017	17/11611	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2017	17/14225	NO	0	CHICK	2017-11	Málaga	NEST	POSITIVE	ITS-D	-	-	NEGATIVE	POSITIVE	POSITIVE	POSITIVE
2017	17/14226	NO	0	CHICK	2017-11	Málaga	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2017	17/14227	NO	0	CHICK	2017-12	Granada	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2017	17/11644	NO	0	CHICK	2017-	Granada	NEST	POSITIVE	MIXED SEQUENCE (ITS-A + ITS-D)	-	-	NEGATI	Nd	POSITIV	NEGATI

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	9				12							VE		E	VE
2017	17/1650	YES	I	CHICK	2017-13	Granada	NEST	POSITIVE	ITS-D	II	C4	NEGATIVE	POSITIVE	POSITIVE	POSITIVE
2017	17/1651	YES	I	CHICK	2017-13	Granada	NEST	POSITIVE	ITS-D	II	C4	POSITIVE	Nd	POSITIVE	POSITIVE
2017	17/2072	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
2017	17/2073	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
2017	17/2074	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	POSITIVE	MIXED SEQUENCE (ITS-A + ITS-D)	-	-	NEGATIVE	Nd	POSITIVE	NEGATIVE
2017	17/2075	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	POSITIVE	ITS-D	-	-	NEGATIVE	POSITIVE	POSITIVE	POSITIVE
2017	17/3719	NO	0	CHICK	2017-15	Granada	NEST	POSITIVE	ITS-D	-	-	NEGATIVE	POSITIVE	POSITIVE	POSITIVE

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2017	17/3721	YES	I	CHICK	2017-14	Toledo	NEST	POSITIVE	ITS-A	VI	-	POSITIVE	Nd	NEGATIVE	POSITIVE
2017	P1V	NO	0	CHICK	2017-25	Alicante	NEST	POSITIVE	ITS-A	VI	-	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE
2016	16/01779	NO	0	CHICK		GREFA	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2016	16/03388	NO	0	CHICK		GREFA	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2016	16/04442	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
2016	16/04433	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2016	16/04478	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
20	16/	NO	0	CHI		UFCS Vendée	CAPTI	NEGATI				NE	NE	NE	NE

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16	0479			CK		(France)	VITY	VE				GATIVE	GATIVE	GATIVE	GATIVE
2016	16/0480	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2016	16/0481	NO	0	CHICK	2016-1	Granada	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2016	16/0482	YES	I	CHICK	2016-2	Granada	NEST	POSITIVE	MIXED SEQUENCE (ITS-A + ITS-D)	VI	A1	POSITIVE	POSITIVE	NEGATIVE	POSITIVE
2016	16/0483	YES	I	CHICK	2016-2	Granada	NEST	POSITIVE	ITS-A	-	-	POSITIVE	POSITIVE	NEGATIVE	POSITIVE
2016	16/0486	NO	0	CHICK	2016-3	Granada	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2016	16/0613	YES	I	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2016	16/06	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGA	Nd	NEGA	NEGA

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	14											TIVE		TIVE	TIVE
2016	16/06/15	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2016	16/06/96	YES	I	CHICK	2016-4	Jaen	NEST	POSITIVE	ITS-D	-	-	NEGATIVE	Nd	POSITIVE	NEGATIVE
2016	16/06/97	NO	0	CHICK	2016-4	Jaén	NEST	POSITIVE	MIXED SEQUENCE (ITS-A + ITS-D)	MIXED (VI+II)	MIXED (A1+C4)	POSITIVE	Nd	POSITIVE	POSITIVE
2016	16/06/98	NO	0	CHICK	2016-5	Almería	NEST	POSITIVE	ITS-A	VI	-	POSITIVE	Nd	NEGATIVE	POSITIVE
2016	16/06/99	NO	0	CHICK	2016-5	Almería	NEST	POSITIVE	FN433473 (ITSIII acording to Grabenstein er et al., 2010)	FN433486 (VIII)	-	NEGATIVE	Nd	POSITIVE	NEGATIVE
2016	16/07/00	YES	I	CHICK	2016-6	Málaga	NEST	POSITIVE	ITS-D	II	C4	NEGATIVE	Nd	POSITIVE	NEGATIVE
2016	16/07/01	YES	I	CHICK	2016-6	Málaga	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE

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2016	16/0702	NO	0	CHICK	2016-7	Granada	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2016	16/0703	NO	0	CHICK	2016-7	Granada	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2016	16/0704	NO	0	CHICK	2016-8	Málaga	NEST	POSITIVE	ITS-D	II	C4	POSITIVE	POSITIVE	NEGATIVE	POSITIVE
2016	16/0705	NO	0	CHICK	2016-8	Málaga	NEST	NEGATIVE				NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
2016	16/0870	NO	0	CHICK	2016-9	Mallorca	NEST	POSITIVE	ITS-D	II	-	NEGATIVE	Nd	POSITIVE	NEGATIVE
2016	16/0871	NO	0	CHICK	2016-9	Mallorca	NEST	POSITIVE	ITS-D	II	A1	NEGATIVE	Nd	POSITIVE	NEGATIVE
2016	16/0922	NO	0	CHICK	2016-10	Jaén	NEST	POSITIVE	ITS-D	II	-	POSITIVE	Nd	NEGATIVE	POSITIVE
20	16/	NO	0	CHI	20	Jaén	NEST	NEGATI				NE	Nd	NE	NE

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16	0923			CK	16-10			VE				GATIVE		GATIVE	GATIVE
2016	16/0924	NO	0	CHICK	2016-12	Cádiz	NEST	NEGATIVE				Nd	Nd	NEGATIVE	Nd
2016	16/0925	NO	0	CHICK	2016-11	Cádiz	NEST	NEGATIVE				NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
2016	16/0972	NO	0	CHICK	2016-13	Mallorca	NEST	POSITIVE	ITS-A	VI	A1	POSITIVE	POSITIVE	POSITIVE	POSITIVE
2016	16/1022	YES	I	CHICK	2016-14	Toledo	NEST	POSITIVE	ITS-A	VI	-	NEGATIVE	Nd	POSITIVE	NEGATIVE
2016	16/1023	YES	I	CHICK	2016-14	Toledo	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2016	16/1191	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2016	16/111	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGA	NEGA	NEGA	NEGA

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	92											TIVE	TIVE	TIVE	TIVE
2016	16/1193	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2016	16/2203	NO	0	CHICK	2016-15	Madrid	NEST	NEGATIVE				Nd	Nd	NEGATIVE	Nd
2016	16/2888	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2016	VR10-16	YES	I	CHICK	2016-25	Valencia	NEST	POSITIVE	ITS-A	-	A1	POSITIVE	POSITIVE	Nd	POSITIVE
2016	VR11-16	YES	I	CHICK	2016-25	Valencia	NEST	POSITIVE	ITS-A	VI	A1	POSITIVE	POSITIVE	Nd	POSITIVE
2016	VR12-16	NO	0	CHICK	2016-16	Alicante	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2016	VR13-16	NO	0	CHICK	2016-1	Alicante	NEST	NEGATIVE				NEGATIV	Nd	Nd	NEGATIV

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					6							E			E
2016	VR14-16	NO	0	CHICK	2016-26	Valencia	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2016	VR15-16	NO	0	CHICK	2016-26	Valencia	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2016	VR17-16	NO	0	CHICK	2016-19	Castelló n	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2016	VR18-16	NO	0	CHICK	2016-19	Castelló n	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2016	VR19-16	NO	0	CHICK	2016-20	Castelló n	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2016	VR20-16	NO	0	CHICK	2016-20	Castelló n	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2016	VR21-16	NO	0	CHICK	2016-21	Castelló n	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2	V	N	0	C	2	Castelló	NES	POS	ITS-D	II	-	P	P	N	P

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016	R22-16	O		HICK	016-21	n	T	ITIVE				OSITIVE	OSITIVE	d	OSITIVE
2016	VR23-16	NO	0	CHICK	2016-18	Valencia	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2016	VR24-16	NO	0	CHICK	2016-18	Valencia	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2016	VR25-16	NO	0	CHICK	2016-24	Alicante	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2016	VR5-16	NO	0	CHICK	2016-22	Alicante	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2016	VR6-16	YES	I	CHICK	2016-22	Alicante	NEST	POSITIVE	ITS-A	-	-	POSITIVE	POSITIVE	Nd	POSITIVE
2016	VR7-16	YES	I	CHICK	2016-17	Valencia	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
201	VR8-	YES	I	CHIC	201	Valencia	NEST	NEGATIVE				NEG	Nd	Nd	NEG

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6	16			K	6-17							ATIVE			ATIVE
2016	VR9-16	NO	0	CHICK	2016-23	Alicante	NEST	POSITIVE	ITS-D	VI	-	POSITIVE	POSITIVE	Nd	POSITIVE
2015	7/10/2015	NO	0	ADULT		Castelló n	ADMISSION WRC	POSITIVE	ITS-D	II	C4	POSITIVE	Nd	Nd	POSITIVE
2015	156/15	YES	III	ADULT		Valencia	ADMISSION WRC	POSITIVE	ITS-A	VI	A2	POSITIVE	POSITIVE	Nd	POSITIVE
2015	15/01556	NO	0	CHICK		GREFA	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/01557	NO	0	CHICK		GREFA	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/03039	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/03400	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIV	Nd	NEGATIV	NEGATIV

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2015	15/04/14	NO	0	CHICK		GREFA	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/04/15	NO	0	CHICK		GREFA	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/04/39	NO	0	CHICK	2015-1	Granada	NEST	POSITIVE	ITS-D	II	C4	NEGATIVE	Nd	POSITIVE	NEGATIVE
2015	15/04/40	NO	0	CHICK	2015-1	Granada	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2015	15/04/41	NO	0	CHICK	2015-2	Almeria	NEST	POSITIVE	ITS-D	-	-	NEGATIVE	Nd	POSITIVE	NEGATIVE
2015	15/04/42	NO	0	CHICK	2015-2	Almeria	NEST	POSITIVE	ITS-D	II	C4	POSITIVE	Nd	POSITIVE	POSITIVE
2015	15/04/43	YES	I	CHICK	2015-3	Granada	NEST	POSITIVE	ITS-A	VI	A1	NEGATIVE	Nd	POSITIVE	NEGATIVE
20	15/	YE	III	CHI	20	Granada	NEST	POSITIV	ITS-A	VI	-	NE	Nd	PO	NE

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15	0444	S		CK	15-3			E				GATIVE		SITIVE	GATIVE
2015	15/0445	YES	I	CHICK	2015-4	Malaga	NEST	POSITIVE	ITS-A	VI	A2	POSITIVE	Nd	POSITIVE	POSITIVE
2015	15/0446	YES	III	CHICK	2015-4	Malaga	NEST	POSITIVE	ITS-A	VI	A2	NEGATIVE	Nd	POSITIVE	NEGATIVE
2015	15/0447	YES	I	CHICK	2015-5	Malaga	NEST	POSITIVE	MIXED SEQUENCE (ITS-A + ITS-D)	MIXED (VI+II)	MIXED (A1+C4)	POSITIVE	Nd	POSITIVE	POSITIVE
2015	15/0449	YES	I	CHICK	2015-6	Almeria	NEST	POSITIVE	ITS-A	VI	A1	POSITIVE	Nd	POSITIVE	POSITIVE
2015	15/0450	YES	I	CHICK	2015-6	Almeria	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/0467	YES	II	CHICK	2015-7	Almeria	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/0590	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE

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2015	15/07/32	YES	I	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/07/33	YES	I	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/07/34	YES	I	CHICK		UFCS Vendée (France)	CAPTIVITY	POSITIVE	ITS-D	II	-	NEGATIVE	Nd	POSITIVE	NEGATIVE
2015	15/08/84	NO	0	CHICK	2015-8	Guadalajara	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/08/85	NO	0	CHICK	2015-9	Toledo	NEST	POSITIVE	ITS-D	II	-	NEGATIVE	Nd	POSITIVE	NEGATIVE
2015	15/11/08	YES	II	CHICK	2015-10	Malaga	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/11/09	YES	II	CHICK	2015-10	Malaga	NEST	POSITIVE	MIXED SEQUENCE (ITS-A + ITS-D)	II	-	NEGATIVE	Nd	POSITIVE	NEGATIVE
20	15/	YE	I	CHI	20	Jaen	NEST	POSITIV	ITS-A	-	-	NE	Nd	PO	NE

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15	1110	S		CK	15-11			E				GATIVE		SITIVE	GATIVE
2015	15/11111	YES	II	CHICK	20155-11	Jaen	NEST	NEGATIVE		-		NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/11112	YES	III	CHICK	20155-12	Jaen	NEST	POSITIVE	ITS-A	VI	A1	NEGATIVE	Nd	POSITIVE	NEGATIVE
2015	15/11113	YES	III	CHICK	20155-12	Jaen	NEST	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/13779	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/13880	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/13881	NO	0	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/113	YES	I	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGA	Nd	NEGA	NEGA

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	82											TIVE		TIVE	TIVE
2015	15/1383	YES	I	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	15/1483	NO	0	CHICK	2015-13	Madrid	NEST	POSITIVE	ITS-D	-	-	NEGATIVE	Nd	POSITIVE	NEGATIVE
2015	15/1484	YES	I	CHICK	2015-13	Madrid	NEST	POSITIVE	ITS-A	VI	A1	POSITIVE	Nd	Nd	POSITIVE
2015	15/1606	YES	I	CHICK		UFCS Vendée (France)	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	NEGATIVE	NEGATIVE
2015	R10/15	NO	0	CHICK	2015-19	Valencia	NEST	NEGATIVE				NEGATIVE	NEGATIVE	Nd	NEGATIVE
2015	R10/15	NO	0	CHICK	2015-20	Valencia	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2015	R11/15	NO	0	CHICK	2015-1	Alicante	NEST	POSITIVE	ITS-A	VI	A1	POSITIVE	POSITIVE	Nd	POSITIVE

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					4										
2015	R12/15	YES	II	CHICK	2015-15	Alicante	NEST	NEGATIVE				NEGATIVE	NEGATIVE	Nd	NEGATIVE
2015	R13/15	YES	I	CHICK	2015-15	Alicante	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2015	R14/15	NO	0	CHICK	2015-21	Valencia	NEST	NEGATIVE				NEGATIVE	NEGATIVE	Nd	NEGATIVE
2015	R17/15	YES	I	CHICK	2015-16	Alicante	NEST	POSITIVE	ITS-D	-	-	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE
2015	R2/15	YES	I	CHICK	2015-22	Valencia	NEST	POSITIVE	MIXED SEQUENCE (ITS-A + ITS-D)	II	-	NEGATIVE	POSITIVE	Nd	POSITIVE
2015	R3/15	YES	II	CHICK	2015-17	Alicante	NEST	POSITIVE	ITS-A	VI	A1	POSITIVE	POSITIVE	Nd	POSITIVE
2015	R5/15	NO	0	CHICK	2015-18	Alicante	NEST	NEGATIVE				NEGATIVE	NEGATIVE	Nd	NEGATIVE
2	R	N	0	C	2	Alicante	NES	NEG				N	N	N	N

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2015	6/15	O		HICK	015-18		T	ATIVE				EGATIVE	EGATIVE	d	EGATIVE
2015	R7/15	NO	0	CHICK	2015-23	Valencia	NEST	NEGATIVE				NEGATIVE	NEGATIVE	Nd	NEGATIVE
2015	R8/15	YES	II	CHICK	2015-23	Valencia	NEST	NEGATIVE				NEGATIVE	NEGATIVE	Nd	NEGATIVE
2015	R9/15	NO	0	CHICK	2015-20	Valencia	NEST	NEGATIVE				NEGATIVE	NEGATIVE	Nd	NEGATIVE
2014	14/00036	NO	0	SUBADULT		Madrid	ADMISSION	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2014	14/02273	NO	0	JUVENILE		Granada	ADMISSION	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2014	14/03220	NO	0	CHICK		GREFA	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2011	14/0	NO	0	CHIC		GREFA	CAPTIVIT	NEGATIVE				NEG	Nd	Nd	NEG

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4	344			K			Y					ATIVE			ATIVE
2014	14/040409	NO	0	CHICK	2014-1	Almeria	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2014	14/041010	NO	0	CHICK	2014-1	Almeria	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2014	14/041111	YES	II	CHICK	2014-2	Malaga	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2014	14/041212	YES	II	CHICK	2014-2	Malaga	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2014	14/054707	YES	I	CHICK	2014-3	Albacete	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2014	14/073404	NO	0	CHICK	2014-4	Jaen	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2014	14/073	YES	I	CHICK	2014-	Jaen	NEST	POSITIVE	ITS-A	VI	A1	POSITIV	POSITIV	Nd	POSITIV

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	5				4							E	E		E
2014	14/09744	YES	II	CHICK	2014-5	Guadalajara	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2014	14/09975	NO	0	CHICK	2014-6	Jaen	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2014	14/09976	NO	0	CHICK	2014-6	Jaen	NEST	POSITIVE	ITS-D	II	C4	POSITIVE	POSITIVE	Nd	POSITIVE
2014	14/09977	YES	II	CHICK	2014-7	Granada	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2014	14/10988	YES	II	CHICK	2014-7	Granada	NEST	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2014	14/13588	YES	I	CHICK	2014-8	Mallorca	NEST	POSITIVE	ITS-A	VI	A1	POSITIVE	POSITIVE	Nd	POSITIVE
2014	14/16115	NO	0	CHICK		GREFA	CAPTIVITY	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
201	14/3	NO	0	JUVE		Granada	ADMISSIO	NEGATIVE				NEG	Nd	Nd	NEG

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4	812			NILE			N					ATIVE			ATIVE
2014	2488/14	NO	0	ADULT		Valencia	ADMISSION WRC	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2014	2489/14	NO	0	ADULT		Valencia	ADMISSION WRC	NEGATIVE				NEGATIVE	Nd	Nd	NEGATIVE
2014	634/14	YES	III	CHICK		Castellon	ADMISSION WRC	POSITIVE	ITS-A	VI	A1	NEGATIVE	POSITIVE	Nd	POSITIVE

Supplementary Table 2. Bonelli's eagles samples from 2014 to 2017. Aptitude: bred in captivity, nestling, admission. Age: C: chick, J: juvenile, SA: subadult, A: adult. L: number of birds with gross lesions compatible with avian trichomonosis. *T. gallinae*: number of birds with a positive result to *T. gallinae* (including culture in TYM medium or PCR from oropharyngeal swabs or culture sediment). N: number of samples. Nests: number of nests in each province. WRC: wildlife recovery centre.

Aptitude (Age)	**Year**	**Region**	**Province**	**N**	**Nests**	***T. gallinae* + (L)**	***T. gallinae* - (L)**
Nestlings (n=132) (C)	2014 (n=13)	Andalucía	Almería	2	1	0	2 (0)
			Granada	2	2	0	2 (0)
			Jaén	4	3	2 (1)	2 (0)
			Málaga	2	1	0	2 (0)

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		Castilla-La Mancha	Albacete	1	1	0	1 (1)
			Guadalajara	1	1	0	1 (1)
		Islas Baleares	Mallorca	1	1	1 (1)	0
	2015 (n=36)	Andalucía	Almería	5	3	3 (1)	2 (2)
			Granada	4	2	3 (2)	1 (0)
			Jaén	4	2	2 (2)	2 (2)
			Málaga	5	3	4 (4)	1 (1)
		Comunidad de Madrid	Madrid	2	1	2 (1)	0
		Castilla-La Mancha	Guadalajara	1	1	0	1 (0)
			Toledo	1	1	0	1 (0)
		Comunidad Valenciana	Alicante	7	5	3 (2)	4 (2)
			Valencia	7	5	1 (1)	6 (1)

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		Andalucía					
			Almería	2	1	2 (0)	0
	2016 (n=44)		Cádiz	2	1	0	2 (0)
			Granada	6	4	2 (2)	4 (0)
			Jaén	4	2	3 (1)	1 (0)
			Málaga	4	2	2 (1)	2 (1)
		Comunidad de Madrid	Madrid	1	1	0	1 (0)
		Castilla-La Mancha	Toledo	2	1	1 (1)	1 (1)
		Comunidad Valenciana	Alicante	6	4	2 (1)	4 (0)
			Castellón	6	3	1 (0)	5 (0)
			Valencia	8	4	2 (2)	6 (2)
		Islas Baleares	Mallorca	3	2	3 (0)	0
	2017 (n=39)	Andalucía	Cádiz	4	3	2 (1)	2 (1)
			Granada	8	5	7 (4)	1 (0)
			Jaén	4	2	1 (0)	3 (0)
			Málaga	2	1	1 (0)	1 (0)
		Castilla-La	Guadalajara	1	1	1 (1)	0

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		Mancha					
			Toledo	1	1	1 (1)	0
		Comunidad Valenciana	Alicante	1	1	1 (0)	0
			Valencia	8	5	2 (2)	6 (0)
			Castellón	7	4	2 (0)	6 (0)
		Islas Baleares	Mallorca	3	2	2 (1)	1 (0)
Captivity (n=48) (C)	2014 (n=3)	GREFA	Madrid	3	-	0	3 (0)
	2015 (n=16)	GREFA	Madrid	4	-	0	4 (0)
		Centre UFCS	Vendée	12	-	1 (1)	11 (5)
	2016 (n=14)	GREFA	Madrid	2	-	0	2 (0)
		Centre UFCS	Vendée	12	-	0	12 (0)
	2017 (n=15)	GREFA	Madrid	3	-	1 (1)	3 (0)
		Centre UFCS	Vendée	11	-	7 (0)	5 (0)
		J-C Morgue	Ardéche	1	-	0	1 (0)
Admission (n=8)	2014	GREFA	Madrid	1 (SA)	-	0	1 (0)
		WRC El Saler	Valencia	2 (A)	-	0	2 (0)
		WRC El	Granada	2 (J)	2	0	2 (0)

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		Blanqueo					
		WRC Forn del Vidre	Castellón	1 (C)	1	1 (1)	0
	2015	WRC El Saler	Valencia	1 (A)	-	1 (1)	0
		WRC Forn del Vidre	Castellón	1 (A)	-	1 (0)	0
Total				188			

Supplementary Table 3. Number of samples positive to culture in TYM or oropharyngeal swab ITS-PCR. Only birds with both analysis were included.

	Swab ITS-PCR +	**Swab ITS-PCR -**	Total
Culture in TYM +	10	9	19
Culture in TYM -	33	74	107
Total	43	83	126

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