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1 **Trichomonad parasite infection in four species of Columbidae in the UK**

2

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12 Running title: *Trichomonas* in British Columbiformes

13

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25 **SUMMARY**

26 *Trichomonas gallinae* is an emerging pathogen in wild birds, linked to recent  
27 declines in finch (Fringillidae) populations across Europe. Globally, the main  
28 hosts for this parasite are species of columbiformes (doves and pigeons); here  
29 we carry out the first investigation into the presence and incidence of  
30 *Trichomonas* in four species of columbiformes in the UK, through live sampling of  
31 wild-caught birds and subsequent PCR. We report the first known UK cases of  
32 *Trichomonas* infection in columbiformes, in 86% of European Turtle Doves  
33 *Streptopelia turtur* sampled, along with 86% of Eurasian Collared Doves  
34 *Streptopelia decaocto*, 47% of Woodpigeons *Columba palumbus* and 40% of Stock  
35 Doves *Columba oenas*. Birds sampled at farms were more likely to be infected if  
36 the farm provided supplementary food for gamebirds. We found three strains of  
37 *T. gallinae* and one strain clustering within the *T. tenax* clade, not previously  
38 associated with avian hosts in the UK. One *T. gallinae* strain was identical at the  
39 ITS/5.8S/ITS2 ribosomal region to that responsible for the finch trichomonosis  
40 epidemic. We highlight the importance of increasing our knowledge of the  
41 diversity and ecological implications of *Trichomonas* parasites in order further to  
42 understand the sub-clinical impacts of parasite infection.

43

44 Keywords: bird-feeders, emerging diseases, farmland birds, population declines,  
45 wildlife management

46

47 **KEY FINDINGS**

- 48 • First recorded cases of trichomonad infection in Turtle Doves in the UK
- 49 • High diversity of parasite strains in pigeons and doves in the UK
- 50 • One strain of parasite clustered within the *Trichomonas tenax* clade, not
- 51 previously found in avian hosts in the UK
- 52 • Parasite incidence was higher on farms providing supplementary food for
- 53 gamebirds
- 54 • One *T. gallinae* strain was identical at the ITS/5.8S/ITS2 ribosomal region
- 55 to the finch trichomonosis strain
- 56

57 **INTRODUCTION**

58 Regrettably, there is a general paucity of studies on sub-clinical disease in wild  
59 bird populations and as a result, disease ecology is not well understood  
60 (Bunbury *et al.* 2008). In the UK, the protozoan parasite *Trichomonas gallinae* is  
61 currently causing widespread declines in finch (Fringillidae) populations  
62 (Robinson *et al.* 2010). Typically, the main hosts of the avian *Trichomonas*  
63 parasite are the Columbiformes (Sansano-Maestre *et al.* 2009), including the  
64 endangered Mauritius Pink Pigeon *Columba mayeri*, where it can be a major  
65 factor in nestling mortality (Bunbury *et al.* 2007), limiting population growth  
66 (Bunbury *et al.* 2008). Currently in the UK, the status of *T. gallinae* infection in  
67 wild dove and pigeon populations is unknown but infection via garden feeders  
68 has been associated with a 35% decline in Greenfinch *Cardualis chloris*  
69 populations within a 12-month period (Robinson *et al.* 2010), and finch  
70 trichomonosis is currently spreading across Europe (Lawson *et al.* 2011a). In  
71 the UK, two species of columbiformes, Collared Doves *Streptopelia decaocto* and  
72 Woodpigeons *Columba palumbus*, commonly host the *T. gallinae* parasite (as  
73 diagnosed through necropsy and microscopic or microbiological confirmation;  
74 Veterinary Laboratories Agency 2009) and also use garden feeders alongside  
75 finches. Recent findings from Lawson *et al.* (2011b) identified the same strain of  
76 trichomonosis in Woodpigeons as in Greenfinches. However these samples were  
77 obtained from only two Woodpigeons that had died as a result of the infection in  
78 2002. As yet there has been no subsequent evidence to suggest (either in  
79 samples from living or deceased birds) that there is a reservoir of finch  
80 trichomonosis within UK columbidae species. Stock Doves *Columba oenas* and  
81 Turtle Doves *Streptopelia turtur* are less likely to feed in garden habitats and to

82 our knowledge there have been only twelve reported cases of *T. gallinae*  
83 infection in Stock Doves between 2002 and 2009, all diagnosed through  
84 examination of clinical histories rather than molecular or microscopic  
85 confirmation of parasite identity (Veterinary Laboratories Agency 2009) and no  
86 reported cases in Turtle Doves within the UK. Indeed, the migratory habits of  
87 Turtle Doves may lead to a reduced exposure to *Trichomonas*, as finch  
88 trichomonosis is strongly seasonal, with the highest rates between September  
89 and February (Robinson *et al.* 2010) when Turtle Doves are migrating or on  
90 wintering grounds.

91

92 *T. gallinae*, the protozoan causative agent of avian trichomonosis, replicates by  
93 binary fission, resulting in the formation of lesions, primarily in the gullet and  
94 respiratory tract, which can lead to death by starvation or suffocation (Stabler  
95 1954; Sansano-Maestre *et al.* 2009; Robinson *et al.* 2010). The parasite itself has  
96 no intermediate host but can be transmitted both horizontally at shared food and  
97 water sources, and in columbiformes vertically through pigeon crop milk which  
98 is fed to young nestlings (Villanúa *et al.* 2006; Bunbury *et al.* 2007). It shows  
99 large genetic variation, with more than 15 different strains belonging to 3 clades  
100 known to infect avian species. Susceptibility and virulence varies between  
101 different strains and as a result <1% of pigeons infected by the *Trichomonas*  
102 parasite display clinical signs (Sansano-Maestre *et al.* 2009). However, sub-  
103 clinical infection can still lead to reduced survival (Bunbury *et al.* 2008) and  
104 prior infection to non-virulent isolates can also confer protection against virulent  
105 isolates (Stabler 1948). This highlights a need for surveillance of wild bird

106 populations that does not rely simply on estimating prevalence by visual  
107 observation of morbidity or mortality.

108

109 Here, we aimed first to establish whether or not *T. gallinae* is present in wild  
110 populations of Turtle Doves, Collared Doves, Woodpigeons and Stock Doves from  
111 farmland sites across East Anglia, where Turtle Dove populations remain at  
112 comparatively high densities. To our knowledge, this is the first study to  
113 investigate the presence of the parasite in dove and pigeon populations in the  
114 UK. Second, we sequenced a subset of positive samples to establish whether or  
115 not *T. gallinae* strains infecting columbiformes sub-clinically are the same as  
116 those causing finch mortality, and to advance understanding of the diversity of *T.*  
117 *gallinae* in UK columbiformes.

118

119

120

121 **MATERIALS AND METHODS**

122 Oral swabs were collected from columbiformes at 12 farmland sites across  
123 Cambridgeshire (1 site near each of Cambourne: 52° 21'N, 0° 06'W; Chrishall:  
124 52° 03'N, 0° 10'E; Witcham: 52° 39'N, 0° 15'E; and Over: 52° 31'N, 0° 01'E), Essex  
125 (1 site near each of Tolleshunt D'Arcy: 51° 77'N, 0° 79'E; Aldham: 51° 89'N, 0°  
126 78'E; Marks Tey: 51° 88'N, 0° 79'E; and Silver End: 51° 85'N, 0° 62'E), Norfolk (2  
127 sites near Hilgay: 52° 56'N, 0° 39'E) and Suffolk (2 sites near Stowmarket: 52°  
128 19'N, 0° 99'E): we restricted sampling to these areas as Turtle Dove numbers are  
129 declining rapidly in the UK and populations are now largely restricted to south-  
130 east England (e.g. Dunn and Morris 2012). Adult birds were caught at temporary  
131 bait sites using whoosh nets and large mesh mist nets (Redfern and Clarke2001)  
132 between June and August 2011; nestlings were temporarily removed from  
133 closely monitored nests, located by searching suitable habitat in areas known to  
134 contain columbiformes. Birds were ringed on the leg using numbered British  
135 Trust for Ornithology metal rings, aged where possible by reference to standard  
136 texts (Baker 1993) and weighed using a digital balance (Satrue, Taiwan, ± 0.1g)  
137 The oral cavity, throat and crop were swabbed using a sterile viscose swab,  
138 which was then inoculated in an individual InPouch TF culture kit (Biomed  
139 Diagnostics, Oregon). The pouches were sealed to avoid cross-contamination,  
140 and incubated at 37°C for at least 72 hours. Previous studies have indicated that  
141 72 hours is sufficient time to allow detection of all *T. gallinae* infections, with no  
142 further infections being detected within a further 4 days (Cover *et al.* 1994; Boal  
143 *et al.* 1998; Bunbury *et al.* 2005). Accordingly, we took 72 hours as a cut-off, after  
144 which subsequent analysis was carried out.

145

146 Parasites were isolated within a fume cupboard, using standard laboratory  
147 procedures to avoid cross-contamination and following the protocol of Riley *et*  
148 *al.* (1992), modified as follows. In brief, 75-100 µl of the culture was centrifuged  
149 at 900 g for 5 min at 4 °C. The resulting pellet was washed twice in 500 µl of  
150 sterile phosphate-buffered saline (PBS) by centrifugation and then re-suspended  
151 in 200 µl of PBS. DNA was extracted from the isolated pellets using a DNeasy  
152 blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's  
153 instructions (Robinson *et al.* 2010).

154

155 Primers TFR1 [f] and TFR2 [r] were used to target the ITS1/5.8S/ITS2 ribosomal  
156 region of the *T. gallinae* protozoan, with an expected product length of 400 bp  
157 (Robinson *et al.* 2010). A positive control sample was obtained from a  
158 Woodpigeon that had visible clinical signs of trichomonosis. A negative control  
159 with molecular grade water in place of DNA was also used in each PCR to confirm  
160 absence of contamination.

161

162 Each PCR reaction consisted of: ~50 ng template DNA; 0.6µM forward and  
163 reverse primers; 1.5 mM MgCl<sub>2</sub>; 0.4 mM dNTPs; 0.5U Go Taq Hot Start  
164 Polymerase (Promega, Madison, WI) and 5X PCR buffer made up to a total  
165 volume of 50 µl with molecular grade water. PCR thermal cycling was conducted  
166 as follows: 5 mins denaturation at 94 °C, then 36 cycles of 1 min at 94 °C, 30 s at  
167 65 °C and 1 min at 72 °C, followed by 5 mins at 72 °C for final elongation (Riley *et*  
168 *al.* 1992). PCR protocols were all carried out on a Gene Amp 9700 PCR system  
169 (Applied Biosystems, Foster City, CA). The PCR products were electrophoresed  
170 through a 0.8 % agarose gel in 0.5x TBE buffer, stained with ethidium bromide

171 and visualised by UV light. All samples from the first PCR were screened again to  
172 confirm the presence or absence of parasites. PCR products were purified using  
173 Wizard SV Gel & PCR Clean-Up System (Promega, Madison, WI) and sequenced  
174 by GATC Biotech (London, UK) or Source BioScience (Nottingham, UK).

175

176 The ITS1/5.8S/ITS2 ribosomal region of rDNA is a reliable species marker for  
177 *Trichomonas* spp., providing evidence of evolutionary pathways (Gaspar Da Silva  
178 *et al.* 2007). This region of rDNA is highly conserved with a low rate of mutation  
179 (Grabensteiner *et al.* 2010) therefore any sequences that were not identical to  
180 existing strains were considered to be a new strain. Forward and reverse  
181 sequences for each PCR product were trimmed and manually aligned, and  
182 assessed for sequencing errors in BioEdit (Hall 2005). The closest matching  
183 sequence to the consensus sequence for each PCR product was determined using  
184 the NCBI-BLAST database (Altschul *et al.* 1997). To construct a phylogenetic  
185 tree, Genbank was searched using the term "*Trichomonas* ITS1", and all  
186 sequences isolated from wild birds (n=33) were aligned with the four unique  
187 sequences from this study, along with representative sequences of *T. tenax*, *T.*  
188 *vaginalis*, *T. canistome*, and *Tetratrichomonas gallinarum*. The outgroup for this  
189 alignment was *Trichomonas foetus* isolate clone 9 (Genbank accession number  
190 DQ243911; Sansano-Maestre *et al.* 2009). ClustalW (Thompson *et al.* 1994) was  
191 used to create a full alignment of the selected sequences, following which any  
192 duplicate sequences were removed so that only unique sequences remained  
193 (n=22). The neighbour joining method was used to create a phylogenetic tree in  
194 MEGA 5.1, with genetic distance measured by the maximum composite  
195 likelihood (Tamura *et al.* 2011). Branch reliability was analysed using a

196 bootstrap of 1000 replicates. To check the reliability of the phylogenetic tree  
197 created using the neighbour joining method, we also constructed a phylogenetic  
198 tree using the minimum evolution method, with genetic distance measured using  
199 maximum parsimony and branch reliability calculated using a bootstrap of 1000  
200 replicates.

201

202 Ecological factors associated with *Trichomonas* infection were examined using a  
203 binomial General Linear Model (GLM) with infection status (positive or negative)  
204 as the response variable. We used the 'dredge' function in the 'MuMIn' (Bartón  
205 2012) package in R (R Core Development Team, 2012) to fit models to all  
206 possible first-level combinations of three explanatory variables we considered  
207 likely to influence *Trichomonas* infection: species, age and gamebird feeder  
208 status (whether or not the farm at each site provided supplementary grain for  
209 gamebirds year-round). Models were ranked using the second-order Akaike's  
210 Information Criteria (AICc), which measures the goodness-of-fit of a model  
211 whilst taking into account the number of variables within each model, and  
212 penalizing models for the addition of variables. Thus, AICc selects models to  
213 maximize the goodness-of-fit whilst retaining the minimum number of  
214 explanatory variables (Burnham and Anderson 2002).

215 **RESULTS**

216 Sixty samples were collected from 14 Turtle Doves, 5 Stock Doves, 7 Collared  
217 Doves and 34 Woodpigeons. 36 samples (60 %) tested positive for *Trichomonas*  
218 infection (Table 1). One top model fitted the data better than all others to  
219 predict *Trichomonas* infection status, when considering a cut off  $\Delta\text{AIC} < 2$   
220 (Burnham and Anderson 2002): the next best model had a  $\Delta\text{AIC}$  of 2.14. The top  
221 model contained all three predictor variables (Table 2). Confidence intervals  
222 for age and gamebird feeder status did not overlap zero, indicating strong  
223 support for the importance of these two variables in influencing *Trichomonas*  
224 infection status (Table 2). Adults were more likely to be infected than nestlings  
225 (Adults 71.4% infected, n=35; Nestlings 44% infected, n=25; Table 2), and birds  
226 sampled at sites providing food for gamebirds were more likely to be infected  
227 than those sampled at sites with no such supplementary feeding (65% infected,  
228 n=40, 6 sites and 50% infected, n=20, 6 sites, respectively; Table 2). Incidence of  
229 infection differed between species, although significant differences as denoted by  
230 non-overlapping confidence intervals were found between only Turtle Dove  
231 (85.7% infected, n=14) and Woodpigeon (47.1% infected, n=34).

232

233 Twenty PCR products were sequenced from 11 Woodpigeons, 9 Turtle Doves  
234 and 1 Stock Dove, yielding 4 unique sequences (Table 3). Both phylogenetic  
235 trees agreed on branch order, and bootstrap estimates for branch reliability  
236 concurred to within 4 % (mean  $\pm$  1 SE of the difference:  $1.00 \pm 0.31$  %). We  
237 present the neighbor joining tree in Figure 1 and the minimum evolution tree in  
238 Appendix 1. Sequence 1 was isolated from 8 individuals, both Woodpigeons and  
239 Turtle Doves, from sites in Essex, Suffolk and Norfolk and was identical to

240 *Trichomonas gallinae* isolate R2505 (Genbank accession number EU881917.1;  
241 Sansano-Maestre *et al.* 2009). Phylogenetic analysis showed Sequence 1 to be  
242 identical to *T. gallinae* strains C, D and E (Lawson *et al.* 2011b), all isolated from  
243 columbiformes in the USA, Spain and Austria, and raptors in Spain and the USA  
244 (Felleisen 1997; Gerhold *et al.* 2008; Sansano-Maestre *et al.* 2009; Grabensteiner  
245 *et al.* 2010), and to fall within the same clade as one strain isolated from  
246 passerines (a presumably captive Canary *Serinus canaria domestica* in Austria;  
247 Figure 1; Grabensteiner *et al.* 2010). Sequence 2 was isolated from 6 individuals:  
248 three Woodpigeons, 2 Turtle Doves and one Stock Dove, from sites in Essex,  
249 Suffolk and Norfolk. This sequence did not match any existing *T. gallinae* strains,  
250 but had 100% query coverage and 100% max identity to *Trichomonas* sp. AP-  
251 2012 isolates EMD-TG2667, EMD-TG2651, PCD-TG2901 and BSD-TG2671  
252 (Genbank accession numbers JQ030996.1, JQ030995.1, JQ030994.1 and  
253 JQ030993.1; A. Peters and S. Raidal, unpublished data). Sequence 2 falls within  
254 the *T. tenax* clade (Figure 1) along with one sequence isolated from humans in  
255 the USA (Felleisen 1997), and one sequence isolated from columbiformes in  
256 Austria (Grabensteiner *et al.* 2010).

257

258 Sequence 3 was isolated from 4 Turtle Doves and one Woodpigeon at three sites  
259 in Essex, and had 100% query coverage and 100% max identity to *T. gallinae*  
260 strain Vienna 5895-C1/06, isolated from a (presumably captive) psittaciforme in  
261 Austria (Genbank accession number JN007005.1; Reinmann *et al.* 2012).

262 Sequence 3 was also identical to *T. gallinae* isolates XT770-05 and XT710-05,  
263 isolated from Greenfinches *Carduelis chloris* and Chaffinches *Fringilla coelebs*  
264 during the finch trichomonosis epidemic (Robinson *et al.*, 2010), along with

265 sequences isolated from columbiformes in Mauritius, Europe and the USA  
266 (Kleina *et al.* 2004; Gaspar Da Silva *et al.* 2007; Gerhold *et al.* 2008; Sansano-  
267 Maestre *et al.* 2009; Grabensteiner *et al.* 2010), raptors in Europe (Sansano-  
268 Maestre *et al.* 2009), and passerines and corvids in the USA (Anderson *et al.*  
269 2009), all classified as *T. gallinae* strain A (Lawson *et al.* 2011b). Sequence 4 was  
270 isolated from the only bird screened that showed any clinical signs of disease, a  
271 Woodpigeon sampled at a site in Essex, with a large caseous yellow lesion in the  
272 oral cavity consistent with trichomonosis. This sequence had 100% query  
273 coverage and 99% max identity to *T. gallinae* isolate P1807 (Genbank accession  
274 number EU881911.1; Sansano-Maestre *et al.* 2009), with two separate base  
275 deletions. Sequences 3 and 4 both fell within the same clade as *T. gallinae* strain  
276 B (Lawson *et al.* 2011b), isolated from raptors in the USA (Gerhold *et al.* 2008).  
277

278 **DISCUSSION**

279 We found the *Trichomonas gallinae* parasite to be present in all four  
280 columbiform species examined, confirming the first cases in Turtle Doves in the  
281 UK, with incidence at 86 %. Whilst our sample size is relatively small, samples  
282 were obtained from a wide geographic area within the current UK range of the  
283 Turtle Dove, suggesting that high levels of infection may be widespread. As we  
284 used molecular methods rather than microscopy to confirm infection, our  
285 approach seems unlikely to report false negatives; however, it is possible that we  
286 may have underestimated true infection rates.

287

288 The overall incidence of *Trichomonas* infection falls within the range found by  
289 other studies: 5.6 % in Mourning Doves *Zenaida macroura* to 92 % in Rock  
290 Pigeons *Columba livia* (Villanúa *et al.* 2006; Sansano-Maestre *et al.* 2009).  
291 Woodpigeons and Stock Doves had much lower incidences of infection than  
292 Turtle Doves and Collared Doves, which both showed higher prevalence than  
293 found in previous studies of these species elsewhere (50% in Turtle Doves in  
294 Spain: Muñoz 1995; 10% for Collared Doves in Iraq: Al-Bakry 2009). Despite this  
295 difference, the incidence in Woodpigeons in our study was 22 % higher than in  
296 mainland Europe (Villanúa *et al.* 2006). This may be an indicator of a general  
297 increase in disease incidence or due to geographical or seasonal variation.  
298 *Trichomonas* in columbiformes tends to be more prevalent during the breeding  
299 season when temperatures are warmer and rainfall lower (Bunbury *et al.* 2007),  
300 partially due to increased stress and bird-bird contact at nesting sites (Sansano-  
301 Maestre *et al.* 2009). In contrast, finch trichomonosis shows highest morbidity

302 and mortality during the winter, although levels of subclinical infection within  
303 this period are unknown (Robinson *et al.* 2010).

304

305 We found higher incidences of *Trichomonas* infection on farms where  
306 supplementary food was supplied for gamebirds than on farms with no  
307 supplementary food. This supports the suggestion that such food sources may  
308 attract high densities of birds, promoting opportunities for disease transmission  
309 and dissemination (e.g. Höfle *et al.* 2004; Lawson *et al.* 2012). Although  
310 introduced gamebirds such as Pheasants *Phasianus colchicus* and Red-Legged  
311 Partridges *Alectoris rufa* are subject to *Trichomonas* parasites (e.g. Pennycott  
312 1998), these species tend to be infected with *Trichomonas gallinarum* rather  
313 than *T. gallinae*. *T. gallinarum* and *T. gallinae* are found within different clades  
314 which suggests that strains may be unlikely to cross between columbiformes and  
315 galliformes at gamebird feeders. Birds in our study were primarily caught in  
316 close proximity to farmyards, which, like garden feeders, may attract sick birds,  
317 especially where supplementary food (such as that for gamebirds) is provided  
318 over extended periods. Our sample may therefore have been biased towards sick  
319 birds with restricted movement. However, all adult Turtle Doves were radio-  
320 tagged (as part of another study) and displayed normal movement patterns,  
321 suggesting no increase in morbidity in this species.

322

323 We found four strains of *Trichomonas* in UK Columbidae. Apart from one strain  
324 isolated from only one Woodpigeon, all strains were found in both Turtle Doves  
325 and Woodpigeons, with one also found in a Stock Dove, suggesting that none are  
326 species-specific, although the examination of additional genes would provide

327 additional corroboration of this. Sequences 1 and 2 were isolated from three  
328 counties of East Anglia, at sites up to 115km apart, suggesting these two strains  
329 are widespread. Sequences 3 and 4 were isolated only from sites in Essex, and  
330 may therefore be more localized, although further work is required to confirm  
331 this. Sequence 1 fell within the same clade as *T. gallinae* sequences from  
332 columbiformes and raptors in Europe and the USA (Felleisen 1997; Gerhold *et al.*  
333 2008; Sansano-Maestre *et al.* 2009; Grabensteiner *et al.* 2010), and fell in the  
334 same clade as one strain isolated from a (presumably) captive Canary in Austria  
335 (Grabensteiner *et al.* 2010). This suggests this clade contains generalist and  
336 widespread avian parasites, supported by the wide geographic spread of this  
337 strain within our study sites. Interestingly, Sequence 1 is identical to a strain  
338 isolated from Collared Doves in their introduced range in the USA (Gerhold *et al.*  
339 2008) suggesting that the apparently widespread nature of this strain might be  
340 linked to the spread of this invasive columbiform. Whilst the majority of UK  
341 columbiformes do not undertake long-distance migration, the exception is the  
342 Turtle Dove, which is a trans-Saharan migrant, providing an additional  
343 mechanism by which *Trichomonas* parasites could be dispersed over large  
344 distances.

345

346 Sequence 2 is of particular interest, as phylogenetically it clusters not with *T.*  
347 *gallinae*, but within the *T. tenax* clade, usually a parasite of humans (Cielecka *et*  
348 *al.* 2000). Grabensteiner *et al.* (2010) found a '*T. tenax*-like' isolate in a Racing  
349 Pigeon *Columba livia* from Austria, and more recently, Peters and Raidal found a  
350 sequence identical to our Sequence 2 in Common Emerald Doves *Chalcophaps*  
351 *indica*, Zebra Doves *Geopelia striata* and Bar-Shouldered Doves *Geopelia*

352 *humeralis* in Australasia (A. Peters and S. Raidal, unpubl. data). Thus, the finding  
353 of this strain in UK columbiformes is not unprecedented, although this suggests  
354 that this strain may be extremely widespread geographically. The Collared Dove  
355 is a relatively recent addition to UK avifauna, spreading from India through a  
356 natural range expansion and it is plausible that this species may have brought  
357 *Trichomonas* strains with it, especially as it is known to carry *Trichomonas*  
358 parasites in its introduced range in North America (Stimmelmayer *et al.* 2012),  
359 along with its native range (e.g. Romagosa and Labisky 2000; Al-Bakry 2009).  
360 However, further analysis of strains across the range of this species would be  
361 required to confirm this. The pathogenicity of this novel strain is unknown (and  
362 it may be a pathogenic strain sampled prior to lesion development): controlled  
363 infections would be required to assess this as prior infection with a non-virulent  
364 strain can lead to sub-clinical infection by a virulent strain that would otherwise  
365 cause clinical signs, confounding correlative observations (Stabler 1948).

366

367 The only bird within our study with macroscopic lesions in the oral cavity at the  
368 time of sampling, was a Woodpigeon that later died as a result of infection.  
369 Although the clinical signs were consistent with trichomonosis (a large caseous  
370 yellow lesion was visible in the oral cavity), no post-mortem was carried out so  
371 the cause of death could not be confirmed, and other lesion-forming diseases  
372 could not be excluded. This bird was infected by Sequence 4, which falls within  
373 the same clade as *T. gallinae* genotype, a strain similar to that responsible for the  
374 finch trichomonosis epidemic in the UK (Lawson *et al.* 2011b). Sansano-Maestre  
375 *et al.* (2009) found that only birds carrying this genotype had visible clinical  
376 signs (referred to as genotype B in this paper), so this outcome runs in

377 accordance with previous findings. A Turtle Dove nestling with clinical signs of  
378 trichomonosis (regurgitated seed and saliva around the beak and a fetid smell,  
379 although no visible oral lesions), was found during September 2011: its sibling  
380 showed no clinical signs and both were depredated prior to fledging. This  
381 nestling was infected by Sequence 3, which falls within the same clade as  
382 Sequence 4. Sequence 3 is identical at the ITS1/5.8S/ITS2 ribosomal region to  
383 that isolated from Greenfinches *Carduelis chloris* and Chaffinches *Fringilla*  
384 *coelebs* during the UK finch trichomonosis epidemic (Robinson *et al.* 2010). It  
385 would be beneficial for further work to examine other functional genes such as  
386 the iron hydrogenase gene, to determine whether this strain is in fact the same  
387 as the epidemic strain (Robinson *et al.* 2010; Lawson *et al.* 2011b). If so, then  
388 this would lend support to the suggestion that the finch trichomonosis epidemic  
389 was a result of parasite spillover from columbiformes to new host species at  
390 shared feeding stations (Robinson *et al.* 2010; Lawson *et al.* 2012). Given that  
391 this nestling showed clinical signs of trichomonosis, further work should also  
392 investigate the potential implications of *Trichomonas* infection for this rapidly  
393 declining dove.

394

395 In the UK, Turtle Doves are a species of particular conservation concern as the  
396 population has declined by 80% between 1995 and 2010 (Risely *et al.* 2012).  
397 During this period of population decline Turtle Doves have halved their number  
398 of nesting attempts per pair, thought to be a result of food stress (Browne and  
399 Aebischer 2003). Compared to other UK columbiformes (that feed on a variety  
400 of weed seeds, buds, shoots and occasionally invertebrates) Turtle Doves are  
401 ecologically unique: firstly in that they rely solely on seed food throughout the

Comment [KCH1]: 'predated'  
means 'occurred at an earlier time'

402 year; and secondly in that they are migratory. Increased agricultural efficiency  
403 has reduced the availability of arable weeds and consequently the seeds upon  
404 which this species relies (Murton *et al.* 1964). This in turn has driven a dietary  
405 switch from weed seeds to cereals and an increased reliance on anthropogenic  
406 food sources such as grain tailings in farmyards (Browne and Aebischer 2003),  
407 which is likely to increase the density of foraging birds and thus increase the  
408 transmission of *Trichomonas* parasites. Increased food stress can decrease  
409 immune function (Lindström *et al.* 2005), inducing chronic stress in birds  
410 (Clinchy *et al.* 2004) and can subsequently increase levels of parasitaemia for  
411 blood parasite infections (Appleby *et al.* 1999). Whether the same mechanism  
412 applies to *Trichomonas* infection is speculative and requires further  
413 investigation. Migratory stress has also been postulated to increase  
414 susceptibility to *Trichomonas* infection (Villanúa *et al.* 2006) and thus may also  
415 contribute to the high levels of infection found in this species.

416

417 In summary, we have provided the first evidence as to the status of *Trichomonas*  
418 infection within Columbidae in the UK. We found a high incidence in both Turtle  
419 Doves and Collared Doves, although our sample size is relatively small. Despite  
420 this, we found a high diversity in parasite strains, with four unique sequences  
421 falling within three different phylogenetic clades: two of *T. gallinae* and one of a  
422 *T. tenax*-like strain, which appears to be geographically widespread. We found a  
423 higher incidence of infection at farms providing food for gamebirds, suggesting  
424 that supplementary feeding may increase disease transmission in farmland  
425 environments (although transmission from gamebirds to Columbidae appears  
426 unlikely), as well as at garden feeders postulated to lead to transmission of finch

427 trichomonosis. One of the sequences isolated from Turtle Doves and  
428 Woodpigeons is identical at the ITS/5.8S/ITS2 ribosomal region to that  
429 responsible for the finch epidemic, although sequencing at other genes is needed  
430 in order to confirm whether this is the same strain. Overall, this work highlights  
431 the need to extend our knowledge of the diversity and ecological implications of  
432 *Trichomonas* parasites to develop effective management strategies for  
433 vulnerable host species.

434

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438

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444

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624

625 Table 1. Incidence and numbers of birds found to be carrying *Trichomonas* in  
626 each species, shown within two age categories. Numbers in the table show %  
627 infected along with total sample size within each species and age group. WP=  
628 Woodpigeon; CD= Collared Dove; SD= Stock Dove; TD= Turtle Dove.

629

630	<b>Incidence (%)</b>	<b>WP</b>	<b>CD</b>	<b>SD</b>	<b>TD</b>
631	Adult	57.9 (19)	86.0 (7)	50.0 (2)	100 (7)
632	Nestling	33.3 (15)	n/a	33.3 (3)	71.4 (7)
633	Total	47.1 (34)	86.0 (7)	40 (5)	85.7 (14)

634

635

636

637 Table 2. Model estimates from the top model examining ecological factors  
638 predicting *Trichomonas* infection. Estimates and 95% CIs for factors are for the  
639 factor stated compares to a reference factor (Age: Adult; Gamebird: Fed; Species:  
640 Collared Dove)  
641

	Estimate	Lower 95% CI	Upper 95% CI
Intercept	2.861	0.320	5.401
Age (Nestling)	-1.547	-2.957	-0.138
Gamebird (Un-Fed)	-1.532	-3.055	-0.009
Species (Stock Dove)	-1.476	-4.490	1.537
Species (Turtle Dove)	0.810	-2.190	3.811
Species (Woodpigeon)	-1.960	-4.479	0.560

642

643 Table 3. Details of sequenced *Trichomonas* samples providing the sequence number from this study, closest Genbank match to each  
644 sample (detailing maximum identity and query coverage), along with the location and age of bird. Species abbreviations are as in the  
645 legend to Table 1. Superscript number following Genbank sequences indicates citation for that sequence, where 1: Sansano-Maestre *et*  
646 *al.* 2009; 2: Peters and Raidal, unpublished data; and 3: Reinmann *et al.* 2012.

647

648	<b>ID</b>	<b>Location</b>	<b>Species</b>	<b>Age</b>	<b>Sequence</b>	<b>Closest Genbank match</b>	<b>Max ident</b>	<b>Query coverage</b>
649	1	Essex	TD	Nestling	1	EU881917.1 <sup>1</sup>	100	100
650	2	Essex	TD	Adult	1	EU881917.1 <sup>1</sup>	100	100
651	3	Essex	WP	Nestling	1	EU881917.1 <sup>1</sup>	100	100
652	4	Essex	WP	Nestling	1	EU881917.1 <sup>1</sup>	100	100
653	5	Norfolk	WP	Adult	1	EU881917.1 <sup>1</sup>	100	100
654	6	Essex	WP	Adult	1	EU881917.1 <sup>1</sup>	100	100
655	7	Essex	WP	Adult	1	EU881917.1 <sup>1</sup>	100	100
656	8	Suffolk	WP	Adult	1	EU881917.1 <sup>1</sup>	100	100
657	9	Essex	TD	Adult	2	JQ030996.1 <sup>2</sup>	100	100

658	10	Essex	TD	Adult	2	JQ030996.1 <sup>2</sup>	100	100
659	11	Suffolk	SD	Nestling	2	JQ030996.1 <sup>2</sup>	100	100
660	12	Norfolk	WP	Adult	2	JQ030996.1 <sup>2</sup>	100	100
661	13	Suffolk	WP	Adult	2	JQ030996.1 <sup>2</sup>	100	100
662	14	Suffolk	WP	Adult	2	JQ030996.1 <sup>2</sup>	100	100
663	15	Essex	TD	Adult	3	JN007005.1 <sup>3</sup>	100	100
664	16	Essex	TD	Adult	3	JN007005.1 <sup>3</sup>	100	100
665	17	Essex	TD	Nestling	3	JN007005.1 <sup>3</sup>	100	100
666	18	Essex	TD	Nestling	3	JN007005.1 <sup>3</sup>	100	100
667	19	Essex	WP	Adult	3	JN007005.1 <sup>3</sup>	100	100
668	20	Essex	WP	Adult	4	EU881911.1 <sup>1</sup>	99	100

669

670

671

672 Figure 1. Phylogenetic analysis using the neighbour joining method and  
673 ITS1/5.8s rRNA/ITS2 sequences of *Trichomonas* spp. found within this study in  
674 comparison to those published in Genbank. Sequences are labelled by Genbank  
675 accession number and *Trichomonas* species/strain. Information in brackets  
676 indicates the species or family from which the strain was isolated along with  
677 geographic location (where available) and a numerical citation. Genetic distance  
678 is by maximum composite likelihood and branch reliability is shown as a  
679 percentage. Sequences obtained from this study are shown as, 'Sequence X'. 0.05  
680 scale bar: substitutions (corrected) per bp. Species abbreviations are as in the  
681 legend to Table 1. Citations are as follows: 1: Grabensteiner *et al.* 2010; 2:  
682 Gerhold *et al.* 2008; 3: Cielecka *et al.* 2000; 4: Felleisen 1997; 5: Xiao *et al.* 2006;  
683 6: Walker *et al.* 2006; 7: Crespo *et al.* 2001; 8: Kutisova *et al.* 2005; 9: Duboucher  
684 *et al.* 2006.

685

686 Figure in separate file

687 Appendix 1. Phylogenetic analysis using the minimum evolution method and  
688 ITS1/5.8s rRNA/ITS2 sequences of *Trichomonas* spp. found within this study in  
689 comparison to those published in Genbank. Sequences are labelled by Genbank  
690 accession number and *Trichomonas* species/strain. Information in brackets  
691 indicates the species or family from which the strain was isolated along with  
692 geographic location (where available) and a numerical citation. Genetic distance  
693 is by maximum composite likelihood and branch reliability is shown as a  
694 percentage. Sequences obtained from this study are shown as, 'Sequence X'. 0.05  
695 scale bar: substitutions (corrected) per bp. Species abbreviations are as in the  
696 legend to Table 1. Citations are as in the legend to Figure 1.

697

698 Figure in separate file

699