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Trichomonad parasite infection in four species of Columbidae in the UK

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Running title: *Trichomonas* in British Columbiformes

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SUMMARY

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

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

KEY FINDINGS

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

INTRODUCTION

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

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

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

MATERIALS AND METHODS

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

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 $MgCl_2$; 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

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

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

RESULTS

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

Twenty PCR products were sequenced from 11 Woodpigeons, 9 Turtle Doves and 1 Stock Dove, yielding 4 unique sequences (Table 3). Both phylogenetic trees agreed on branch order, and bootstrap estimates for branch reliability concurred to within 4 % (mean ± 1 SE of the difference: 1.00 ± 0.31 %). We present the neighbor joining tree in Figure 1 and the minimum evolution tree in Appendix 1. Sequence 1 was isolated from 8 individuals, both Woodpigeons and 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, JQ0309941 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

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

DISCUSSION

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

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

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

Sequence 2 is of particular interest, as phylogenetically it clusters not with *T. gallinae*, but within the *T. tenax* clade, usually a parasite of humans (Cielecka *et al.* 2000). Grabenesteiner *et al.* (2010) found a '*T. tenax*-like' isolate in a Racing Pigeon *Columba livia* from Austria, and more recently, Peters and Raidal found a sequence identical to our Sequence 2 in Common Emerald Doves *Chalcophaps 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 (Stimmelmayr *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

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

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

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

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

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

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

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624

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

Incidence (%)	**WP**	**CD**	**SD**	**TD**
Adult	57.9 (19)	86.0 (7)	50.0 (2)	100 (7)
Nestling	33.3 (15)	n/a	33.3 (3)	71.4 (7)
Total	47.1 (34)	86.0 (7)	40 (5)	85.7 (14)

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

10	Essex	TD	Adult	2	JQ030996.1[2]	100	100
11	Suffolk	SD	Nestling	2	JQ030996.1[2]	100	100
12	Norfolk	WP	Adult	2	JQ030996.1[2]	100	100
13	Suffolk	WP	Adult	2	JQ030996.1[2]	100	100
14	Suffolk	WP	Adult	2	JQ030996.1[2]	100	100
15	Essex	TD	Adult	3	JN007005.1[3]	100	100
16	Essex	TD	Adult	3	JN007005.1[3]	100	100
17	Essex	TD	Nestling	3	JN007005.1[3]	100	100
18	Essex	TD	Nestling	3	JN007005.1[3]	100	100
19	Essex	WP	Adult	3	JN007005.1[3]	100	100
20	Essex	WP	Adult	4	EU881911.1[1]	99	100

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

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

Figure in separate file