

1 1. TITLE PAGE

2 Endemic infection of the common mynah *Acridotheres tristis* with *Trichomonas gallinae* the agent of
3 avian trichomonosis.

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14 (Short informative running title.)

15 *Trichomonas gallinae* infection of mynah.

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19 2. SUMMARY

20

21 Avian trichomonosis is an architypal disease of wild columbids and those birds that predate them.
22 Increasingly though, it has been reported in passerines; a recent and ongoing epidemic in the
23 chaffinches and greenfinches of Europe and outbreaks amongst house finches, American
24 goldfinches and purple finches in North America. The parasite, *Trichomonas gallinae*, causes
25 lesions in the upper respiratory tract which can cause mortality associated with dehydration and
26 emaciation. This paper reports for the first time, the widespread, endemic and often
27 asymptomatic infection of common mynah (*Acridotheres tristis*) around the Faisalabad District,
28 Pakistan. Parasite typing was used to investigate the potential for transmission among the
29 frequent sympatric species. Type C parasites were found in mynah, and while this is analagous to
30 the pandemic finch strain which is Type A, it is the first known example of passerine infections of
31 this parasite genotype. Subtype analysis showed the strain to be C4 a subtype which has a
32 widespread distribution in columbids.

33

34 *Key words:*

35 Trichomonosis, Trichomoniasis, *Trichomonas gallinae*, subtyping, passerine, sturnidae, mynah,
36 columbid, ITS1/5.8S/ITS2, Fe-hydrogenase

37

38 KEY FINDINGS

- 39 • The common mynah is susceptible to *Trichomonas gallinae* which is normally a
40 disease of pigeons and doves, but which has caused dramatic species declines in
41 European finches in recent years.
- 42
- 43 • Common mynah infection is widespread and frequent in the area surveyed, but
44 appears to cause little pathology suggesting the common mynah as a previously
45 undetected reservoir for this parasite.
- 46
- 47 • Only one genotype of parasite was found, the is different to the one affecting
48 European finches indicating that the common mynah infections are not linked to
49 the finch epidemic.

50

51 3. INTRODUCTION

52 The flagellated protozoan parasite *Trichomonas gallinae* is an etiological agent of
53 Avian trichomonosis, ordinarily affecting the upper respiratory and gastrointestinal tract
54 of birds. It is frequently reported in columbids and birds of prey and is also known as
55 cankar and frounce. Infected birds generally present with esophageal lesions, lethargy,
56 difficulty in breathing, watery eyes, drooling and wet feathers.

57 Columbiformes are beleived to be the primary reservoir host of this parasite, with
58 *Columba livia* considered as an efficient transmitters of *T. gallinae* throughout the world
59 (Forrester & Foster, 2008; Stabler, 1954). The disease is a conservation concern for
60 endangered columbids such as the pink pigeon (Bunbury *et al.*, 2007) and migratory
61 columbids such as the Turtle dove have the potential to spread the parasite over long
62 distances (Stockdale *et al.*, 2015). Several migratory passerine species, which are host to

63 this disease, such as the greenfinch *Chloris Chloris* and chaffinch *Fringilla Coelebs* which
64 have been linked to trichomonosis during their migratory behaviour (Lawson *et al.*,
65 2011b).

66 In recent years the genotype, prevalence and host range of *Trichomonas gallinae*
67 has been subject to significant scrutiny in Europe (Chi *et al.*, 2013; Ganas *et al.*, 2014;
68 Lawson *et al.*, 2006; Lawson *et al.*, 2011a; Lawson *et al.*, 2011b; Robinson *et al.*, 2010; Zu
69 Ermgassen *et al.*, 2016) and North America (Anderson *et al.*, 2009; McBurney *et al.*, 2015),
70 however, in many other parts of the world the distribution of genotypes of *Trichomonas*
71 *gallinae* remains largely uninvestigated with no major studies reported from Asia and the
72 Indian subcontinent. This is a region where columbid populations such as the blue rock
73 pigeon (*Columba livia*) occur in sympatric flocks and share roost with other avian species.
74 Notably, the common mynah, *Acridotheres tristis*, is a passerine species described as the
75 most invasive of all avian species. It occurs in mixed roosts with *Columba livia* and this
76 close contact suggests that the species may share pathogens. Indeed it has been suggested
77 that the invasive nature of *Acridotheres tristis* and its ability to adapt to new niches has
78 meant that it is susceptible to a wide variety of parasite infections (Clark *et al.*, 2015) and
79 we considered whether it might be susceptible to infection with *Trichomonas gallinae* and
80 so might possess the potential to act a shuttling vector of avian trichomonas between the
81 main *Columba livia* reservoir and other ecological niches to which it is able to adapt with
82 increased exposure of infected birds to avian livestock being of particular concern.

83 The common mynah belongs to the order Passeriformes and like the starlings
84 belong to the family sturnidae. It is considered omnivorous, and constructs often large
85 but mostly temporary roosts close to human habitation; inhabiting rural and urban
86 gardens, cultivations among the university and college campuses and the road side light
87 vegetation. The mynah has a relatively short home range of about three to five kilometers

88 during its daily diurnal visitations. It intensively feeds on figs and seeds, unripe fruits,
89 insects, small size frogs and snakes but may also take small, juvenile and dead birds on
90 occasion.

91 Our study was based in the Indian subcontinent where *Acridotheres tristis* is native,
92 from 20km radius of Faisalabad. Faisalabad is the third most populous city in Pakistan,
93 the second largest in the eastern province of Punjab and its latitude and longitude
94 coordinates are 31.42°, 73.09°. The surrounding countryside, irrigated by the lower
95 Chenab River, produces cotton, wheat, sugarcane, vegetables fruits and tree plantations
96 dominated by *Salmlia malabarica*, *Terminalia arjuna*, *Cedrella toona*, *Dalbergia sissoo*,
97 *Ficus bengalensis*, *Eucalyptus* species, *Mangifera dactylifera*. Faisalabad has been
98 classified as a hot desert climate (BWh) by the Köppen-Geiger climate classification
99 system. In this region eight different sites were identified and selected with sympatric
100 flocks of mynah and blue pigeons, i.e., University of Agriculture Faisalabad (UAF), Gatwala
101 Forest plantation (GAT), Postgraduate agricultural research station (PARS), Amipur
102 Canal Rest House (AMI), Satina Canal Rest House (SAT), Burala Canal Rest House (BRU),
103 Tarkhani Canal Rest House (TAR) and Moongi Canal Rest House (MON). These study sites
104 are divided into three categories, i.e., urban (UAF, GAT), semi urban (PARS, AMI, SAT) and
105 rural (BRU, TAR, MON) (Figure 1).

106

107 4. MATERIALS AND METHODS

108

109 **Ethical Approval**

110 This work was approved by the Directorate of Graduate Studies and Research
111 Board as permissible by the University of Agriculture, Faisalabad ethics committee. The
112 approval number was 37195-98, dated 31.12.13.

113

114 **Screening of mynah for *Trichomonas gallinae***

115 The mynahs were captured (N=167), from different locations of Central Punjab
116 with the mist nets. The nets were erected straight into the respective fields, close to the
117 trees viz, *Salmalia malabarica*, *Terminalia arjuna*, *Cedrella toona*, *Dalbergia sissoo*, *Ficus*
118 *bengalensis*, *Eucalyptus* species, which served as a roost to the majority of birds. The nets
119 were carefully watched and mostly the mynahs were captured during the late evening
120 hours; the time they return to roost. The captured population contained 75 males and 92
121 females from different locations of Faisalabad district were maintained in an aviary in the
122 department (Zoology, Wildlife and Fisheries). Upon capture, birds were evaluated
123 clinically on the basis of their body weight (g), wing length (mm), tarsus length (mm), tail
124 length (mm), beak length (mm) and head circumference (mm). Birds were then screened
125 for *T. gallinae* as follows: A sterile calcium-alginate cotton swab tip was inserted gently
126 within the oral cavity of the common mynah to obtain the saliva coated swabs. The swabs
127 were used to inoculate InPouch™ TV culture packs (BioMed Diagnostics, USA) according
128 to the manufacturer's instructions (BioMed Diagnostics, Santa Clara, California, USA).
129 Cultures were incubated (37°C) to assess the growth performance of the parasite (*T.*
130 *gallinae*) for a period of 7-10 days. Trichomonosis cases confirmed microscopically from
131 culture were subjected to the DNA extraction and subsequently genotyped.

132

133 **Genotyping of Mynah isolates**

134 *Trichomonas gallinae* DNA was obtained from the parasite culture using QIAGEN
135 mini kit according to the manufacturer's instructions (QIAGEN, Valencia, California). We
136 have previously proposed a simple binomial sequence based genotyping system for
137 *Trichomonas gallinae* based on the ITS region and Iron dehydrogenase (*FeHyd*) gene

138 sequences which is now widely adopted and undertaken routinely. Genotyping was
139 undertaken essentially as previously described (Chi *et al.*, 2013). Briefly, DNA from *T.*
140 *gallinae* cultures were subjected to ITS region amplification using TFR1
141 (TGCTTCAGTTCAGCGGGTCTTCC) and TFR2 (CGGTAGGTGAACCTGCCGTTGG) primers. A
142 fragment of the Fe-hydrogenase gene was also amplified from positive samples TrichhydFOR
143 (GTTTGGGATGGCCTCAGAAT) and TrichhydREV (AGCCGAAGATGTTGTCTGAAT) primers.
144 Negative (water) and positive (purified *T. gallinae* DNA from an infected greenfinch) controls
145 were included in each PCR run. All amplicons were directly sequenced using a commercial
146 service (Source BioScience, Nottingham, UK).

147

148 5. RESULTS

149 ***Trichomonas gallinae* is a common endemic infection of *Acridotheres tristis***

150 Based on 33 culture positives from 167 birds screened, we approximate the
151 regional prevalence of *Trichomonas gallinae* to be 20%. Of those 33 birds that did culture
152 positive just 11 exhibited markers of poor health/conditions and in just 4 cases were
153 lesions in the upper digestive tract observable with most infected birds appearing to
154 carry the infection asymptotically (Table 1). We found infected mynah in each of the
155 study locations (Fig. 2) indicating that the infection is widespread in this region.
156 Interestingly, although at least some mynah were infected in each of the study sites, most
157 of the infections detected were of male birds and the number of cases at BRU and AMI
158 where contact between common mynah and blue pigeon is the most limited also showed
159 the lowest infection rates.

160

161 ***Trichomonas gallinae* isolates from *Acridotheres tristis* are all of the C4 subtype**

162 Genotypic analysis indicated that all the common mynah isolates among the
163 Faisalabad samples were shared the same genotype. Of the 33 sequences procured from
164 mynah, all were identified to be *T. gallinae* (ribo)Type C by the finding that their
165 sequences were identical to the Type C reference sequence EU215362. Similarly all of the
166 sequences obtained from the *FeHyd* amplicons were identical to the reference sequence
167 KC529662 An illustrative phylogenetic tree is provided to show the evolutionary
168 relationship of these isolates and other avian isolates which have previously been
169 characterised (Fig. 4). The C4 genotype has previously been identified in UK and USA
170 columbids (AA and KMT, unpublished) but the first description of this genotype from
171 asia/indian-subcontinent or in a passerine species.

172

173 6. DISCUSSION

174 **A new genotype of *Trichomonas gallinae* in passerine birds**

175 Our study reports the highest prevalence for *T. gallinae* carriage recorded in any non
176 columbid species and is the first example of a host species other than columbids in which
177 the parasite is widely carried without apparent pathology. It is striking because all
178 genotyped isolates associated with outbreaks of passerine disease have previously been
179 caused by the A1 genotype which is associated with considerable pathogenicity and
180 mortality, particularly in passerine hosts. In this study C4, a genotype that is less
181 associated with such virulence characteristics than A1, is prevalent in live, asymptomatic
182 birds suggesting that mynah has the potential to be a vector for this parasite between the
183 main columbid reservoir and to niches which ordinarily would be isolated from it. It is
184 also interesting that in the UK where an epidemic caused by the A1 genotype finch strain
185 of *T. gallinae* has been taking place since 2006, starlings (a very common species in the
186 same family as the mynah, the Sturnidae) have not been one of at least 12 passerine

187 species diagnosed with the disease by the Garden Wildlife Health project, a national wild
188 bird disease surveillance programme (B. Lawson, personal communication).

189

190 **Mynah - as a new reservoir or a dead end host?**

191 On the basis of this study, we conclude that the common mynah has the potential
192 to serve as a reservoir for avian trichomonosis throughout the study sites of Faisalabad, Pakistan
193 and perhaps throughout its entire (tropical and subtropical) range. This finding is particularly
194 concerning because of the recognized ability of mynah to adapt to new niches and particularly
195 ones from which columbids may normally be excluded extending the potential for exposure of
196 susceptible domestic and wild avifauna to infection. Transmission of influenza from free living
197 mynah to farmed poultry has previously been demonstrated (Body *et al.*, 2015) and similarly our
198 observation of mynah infection by a known pathogen of avian livestock will be of particular
199 concern to poultry producers where mynah may gain access to flocks. Nevertheless, the
200 observations in this study do not in any way address transmission to and from the mynah as a
201 host. We cannot yet speculate on whether the infections observed are spillover from sympatric
202 columbids or passed within the species by other mynah. Certainly, mynah feed in sympatric
203 flocks with blue rock pigeons throughout this region and so may be infected from them. It is also
204 not clear whether mynahs pass the infections to partners and young or even whether the
205 infection is normally pathogenic, fatal or resolves. It is also not clear why there is only one subtype
206 discernable, whether susceptibility is limited to this subtype, whether it represents a mynah
207 adapted strain or whether the pathogenicity of other subtypes means they are quickly eliminated
208 from mynah populations when transmissions do occur. These intriguing questions are though
209 tractable and answering them will be important in gauging the risk posed by mynahs to other
210 susceptible avian populations.

211

212 7. ACKNOWLEDGEMENTS

213 We thank Diana Bell, Becki Lawson, Andrew Cunningham and Johanna Nader for helpful
214 and generous discussion and feedback.

215

216 8. FINANCIAL SUPPORT

217 This study was financially supported by IRSIP project of the Higher Education
218 Commission, Pakistan which included a travel to and support for HF while at the
219 University of East Anglia. AA was supported by an award from the Saudi Arabian Cultural
220 Embassy, London and by the King Saud University in Riyadh, Saudi Arabia.

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223 9. REFERENCES

- 224 **Anderson, N. L., Grahn, R. A., Van Hoosear, K. and BonDurant, R. H.** (2009). Studies of trichomonad
225 protozoa in free ranging songbirds: prevalence of *Trichomonas gallinae* in house finches
226 (*Carpodacus mexicanus*) and corvids and a novel trichomonad in mockingbirds (*Mimus*
227 *polyglottos*). *Veterinary parasitology*, **161**, 178-186.
- 228 **Body, M. H., Alrarawahi, A. H., Alhubsy, S. S., Saravanan, N., Rajmony, S. and Mansoor, M. K.**
229 (2015). Characterization of Low Pathogenic Avian Influenza Virus Subtype H9N2 Isolated
230 from Free-Living Mynah Birds (*Acridotheres tristis*) in the Sultanate of Oman. *Avian Diseases*,
231 **59**, 329-334.
- 232 **Bunbury, N., Jones, C. G., Greenwood, A. G. and Bell, D. J.** (2007). *Trichomonas gallinae* in Mauritian
233 columbids: implications for an endangered endemic. *J Wildl Dis*, **43**, 399-407. doi:
234 10.7589/0090-3558-43.3.399.
- 235 **Chi, J. F., Lawson, B., Durrant, C., Beckmann, K., John, S., Alrefaei, A. F., Kirkbride, K., Bell, D. J.,**
236 **Cunningham, A. A. and Tyler, K. M.** (2013). The finch epidemic strain of *Trichomonas*
237 *gallinae* is predominant in British non-passerines. *Parasitology*, **140**, 1234-1245.
- 238 **Clark, N. J., Olsson-Pons, S., Ishtiaq, F. and Clegg, S. M.** (2015). Specialist enemies, generalist
239 weapons and the potential spread of exotic pathogens: malaria parasites in a highly invasive
240 bird. *Int J Parasitol*, **45**, 891-899. doi: 10.1016/j.ijpara.2015.08.008.
- 241 **Forrester, D. J. and Foster, G. W.** (2008). Trichomonosis. *Parasitic diseases of wild birds*, 120-153.
- 242 **Ganas, P., Jaskulska, B., Lawson, B., Zadavec, M., Hess, M. and Bilic, I.** (2014). Multi-locus
243 sequence typing confirms the clonality of *Trichomonas gallinae* isolates circulating in
244 European finches. *Parasitology*, **141**, 652-661. doi: 10.1017/S0031182013002023.
- 245 **Lawson, B., Cunningham, A., Chantrey, J., Hughes, L., Kirkwood, J., Pennycott, T. and Simpson, V.**
246 (2006). Epidemic finch mortality. *Veterinary Record*, **159**, 367-367.

247 **Lawson, B., Cunningham, A. A., Chantrey, J., Hughes, L. A., John, S. K., Bunbury, N., Bell, D. J. and**
248 **Tyler, K. M.** (2011a). A clonal strain of *Trichomonas gallinae* is the aetiologic agent of an
249 emerging avian epidemic disease. *Infection, Genetics and Evolution*, **11**, 1638-1645.

250 **Lawson, B., Robinson, R. A., Neimanis, A., Handeland, K., Isomursu, M., Agren, E. O., Hamnes, I. S.,**
251 **Tyler, K. M., Chantrey, J. and Hughes, L. A.** (2011b). Evidence of spread of the emerging
252 infectious disease, finch trichomonosis, by migrating birds. *EcoHealth*, **8**, 143-153.

253 **McBurney, S., Kelly-Clark, W. K., Forzan, M. J., Lawson, B., Tyler, K. M. and Greenwood, S. J.** (2015).
254 Molecular characterization of *Trichomonas gallinae* isolates recovered from the Canadian
255 Maritime provinces' wild avifauna reveals the presence of the genotype responsible for the
256 European finch trichomonosis epidemic and additional strains. *Parasitology*, **142**, 1053-1062.
257 doi: 10.1017/S0031182015000281.

258 **Robinson, R. A., Lawson, B., Toms, M. P., Peck, K. M., Kirkwood, J. K., Chantrey, J., Clatworthy, I.**
259 **R., Evans, A. D., Hughes, L. A., Hutchinson, O. C., John, S. K., Pennycott, T. W., Perkins, M.**
260 **W., Rowley, P. S., Simpson, V. R., Tyler, K. M. and Cunningham, A. A.** (2010). Emerging
261 Infectious Disease Leads to Rapid Population Declines of Common British Birds. *PLoS ONE*, **5**.
262 doi: ARTN e1221510.1371/journal.pone.0012215.

263 **Stabler, R. M.** (1954). *Trichomonas gallinae*: a review. *Experimental Parasitology*, **3**, 368-402.

264 **Stockdale, J. E., Dunn, J. C., Goodman, S. J., Morris, A. J., Sheehan, D. K., Grice, P. V. and Hamer, K.**
265 **C.** (2015). The protozoan parasite *Trichomonas gallinae* causes adult and nestling mortality
266 in a declining population of European Turtle Doves, *Streptopelia turtur*. *Parasitology*, **142**,
267 490-498. doi: 10.1017/S0031182014001474.

268 **Zu Ermgassen, E. K., Durrant, C., John, S., Gardiner, R., Alrefaei, A. F., Cunningham, A. A. and**
269 **Lawson, B.** (2016). Detection of the European epidemic strain of *Trichomonas gallinae* in
270 finches, but not other non-columbiformes, in the absence of macroscopic disease.
271 *Parasitology*, **143**, 1294-1300. doi: 10.1017/S0031182016000780.

272

273 Table 1: Occurrence of different strains (*Trichomonads*) as isolated from the available
 274 bird species based on *FeHyd* gene with their GenBank reference.
 275

Host species	Type	Origin	GenBank
Greenfinch	A1	UK	JF681136
Sparrow hawk	A1.1	UK	KC529660
Wood pigeon	A1.2	UK	KC962158
Wood pigeon	A1.3	UK	KC529661
Madagascar turtle dove	A2	Seychelles	JF681141
Rock pigeon	C1	North America	AF446077
Wood pigeon	C2	UK	KC529664
Rock pigeon	C3	UK	KC529663
Wood pigeon	C4	UK	KC529662
Feral pigeon	C8	UK	KY569256
Feral pigeon	C9	UK	KY569257
Socorro dove	C10	UK	KY569258
Tawny owl	C11	UK	KY569259

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Table 2: Characteristic lesions (Trichomonosis) of common mynah consistent with culture using the ITS region and *FeHyd* sequence.

#	Case ID	SEX	Clinical Signs	Lesions	Culture	ITS	<i>FeHyd</i>
1	S1A4Y4	M	+	-	-	C	C4
2	S1A8Y8	M	-	-	+	C	C4
3	S1A1B1	F	+	+	+	C	C4
4	S1A6Y6	M	-	-	+	C	C4
5	S1A8Y3	M	-	-	+	C	C4
6	S2A3G3	F	-	-	+	C	C4
7	S2A3O3	M	-	-	+	C	C4
8	S1A1O1	M	-	-	+	C	C4
9	S2A6O6	F	+	-	+	C	C4
10	S2A10O10	M	-	-	+	C	C4
11	S3A4BL4	M	-	-	+	C	C4
12	S3A8W3	F	-	-	+	C	C4
13	S4A9BR9	M	-	-	+	C	C4
14	S4A7BR7	M	-	-	+	C	C4
15	S4A8P8	M	-	-	+	C	C4
16	S5A8Y8	F	-	-	+	C	C4
17	S5A2B2	M	+	-	+	C	C4
18	S5A6Y2	F	-	+	+	C	C4
19	S5A8Y3	F	-	+	+	C	C4
20	S5A9Y9	M	-	-	+	C	C4
21	S6A8G8	F	-	-	+	C	C4
22	S6A3O3	M	-	-	+	C	C4
23	S6A6O6	F	+	-	+	C	C4
24	S6A9G7	F	-	-	+	C	C4
25	S7A7W7	M	+	-	+	C	C4
26	S7A3BL3	F	-	-	+	C	C4
27	S7A5BL5	M	-	-	+	C	C4
28	S8A10BR10	M	-	-	+	C	C4
29	S8A5A5	M	-	-	+	C	C4
30	S7A4A4	M	-	-	+	C	C4
31	S8A9BR4	M	+	+	+	C	C4
32	S8A10BR5	F	+	-	+	C	C4

280 Figure 1: *T. gallinae* is a frequent infection of common mynah (*Acridotheres tristis*) across
281 the Faisalabad district. The map shows eight different ecological study sites. Prevalence
282 of infection is indicated as the proportion of birds captured and released at each site.
283 Overall prevalence is also noted. University of Agriculture Faisalabad=UAF, Gatwala
284 Forest Plantation=GAT, Postgraduate Agricultural Research Station=PARS, Amipur Canal
285 Rest House=AMI, Satina Canal Rest House =SAT, Burala Canal Rest House =BRU, Trkhani
286 Canal Rest House =TAR and Moongi Canal Rest House = MON.

287

288 Figure 2: *T. gallinae* phylogeny using the neighbour-joining method for the *FeHyd* region
289 from the sequences described in Table 2. *Trichomonas vaginalis* is included as an
290 outgroup. The bootstrap consensus tree was constructed using the Tamura–Nei model
291 and 2000 replicates. Tree topology was tested using 719 positions. †Finch Strain ‡Mynah
292 Isolates

293