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1 TITLE

2 ***Eimeria* spp. in captive-reared corncrakes (*Crex crex*): GeneScan assay results**  
3 **consistent with high prevalence of infection and extra-intestinal life stages**

4

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18 **ABSTRACT**

19 *Eimeria crecis* and *Eimeria nenei* have been detected in association with enteric disease  
20 ('coccidiosis') in the corncrake (*Crex crex*: Family Rallidae, Order Gruiformes). Both parasite  
21 species are common in apparently healthy free-living corncrakes, but captive-bred juvenile  
22 birds reared for reintroduction appeared particularly susceptible to clinical coccidiosis. We  
23 investigated the occurrence and relative pathogenicity of these *Eimeria* species in this  
24 juvenile corncrake population and developed a diagnostic species-specific polymerase chain  
25 reaction (PCR) for their identification. PCR amplification and sequencing of 18S rDNA was  
26 performed on genomic DNA extracted from samples of corncrake intestine, liver and spleen.  
27 Sequences generated were used to design a GeneScan diagnostic PCR assay targeting a  
28 species-specific TTA indel located within the 18S rDNA – the results suggested this assay  
29 was more sensitive than the 18S rDNA/amplicon sequencing approach. *Eimeria* sp. DNA  
30 (consistent with *Eimeria* sp. infection) was detected at a high prevalence and *E. crecis* was  
31 the predominant species. Each *Eimeria* species was detected in cases with and without  
32 histological evidence of coccidiosis: parasite detection was not statistically associated with  
33 disease. In addition to intestinal tissue, liver and spleen samples were positive for *Eimeria*  
34 sp. DNA. Its detection in tissues other than intestine is unusual and a novel finding in  
35 corncrakes, although extra-intestinal infection occurs with closely-related *Eimeria* species in  
36 cranes (Family Gruidae, Order Gruiformes). *Eimeria* sp. infection of corncrakes appears  
37 typically to be chronic, and to exhibit extra-intestinal spread: as for cranes, these  
38 characteristics may be adaptations to the host's migratory nature.

39

40 **Research highlights**

- 41 • High prevalence of *Eimeria* sp. (*E. crecis* significantly more common than *E. nenei*)
- 42 • Detection of *E. crecis* and *E. nenei* DNA in corncrake spleen and liver tissue
- 43 • Improved *Eimeria* spp. detection through development/application of a GeneScan
- 44 assay.

## 45 **Introduction**

46 The corncrake (*Crex crex*) (Family Rallidae, Order Gruiformes) is a migratory rail that inhabits  
47 tall vegetation in meadows and grasslands. The species breeds across Europe and Central  
48 Asia, spending winter months in sub-Saharan Africa (Schaffer & Green, 2001). Though global  
49 populations are classified as being of Least Concern (IUCN, 2012), breeding populations in  
50 Western Europe have declined significantly in both number and range over the past century  
51 (Koffijberg & Schaffer, 2006). The decline in Western Europe has largely been attributed to  
52 changes in farming practices including the introduction of mechanised agriculture and a  
53 movement from hay to silage harvests, both of which typify more intensive grassland  
54 management systems (Green *et al.*, 1997). Following conservation efforts initiated in 1992,  
55 corncrake populations in the Scottish Hebridean islands and Ireland continue to breed  
56 successfully, however with a very limited range the species remains vulnerable to extinction  
57 in the UK (O'Brien *et al.*, 2006).

58 A corncrake reintroduction project (CRP) was initiated in 2001, with the aim of restoring  
59 corncrakes to the wild in eastern England through establishment of a viable breeding  
60 population (Carter & Newbery, 2004). Partners in the initiative (which has been scaled back  
61 since 2017) have included the Zoological Society of London (ZSL), the Royal Society for the  
62 Protection of Birds (RSPB), Natural England (NE) and Pensthorpe Conservation Trust (PCT).  
63 Corncrake chicks were captive-bred, reared to 10-14 days of age in quarantine facilities at  
64 ZSL Whipsnade Zoo (WZ) and PCT, and then transferred to pre-release pens close to the  
65 release site, in which they underwent a three-week acclimatisation period prior to release  
66 (Carter & Newbery, 2004). All birds underwent a clinical examination (health check) before  
67 transfer to the pre-release pens and also before final release. Only birds considered as healthy  
68 were transferred and released.

69 The *Eimeria* species (phylum: Apicomplexa) are highly host-specific protozoan parasites  
70 which are closely related to *Toxoplasma gondii*. *Eimeria* species have traditionally been  
71 characterised based upon sporulated oocyst morphology (e.g. Long *et al.*, 1976). The

72 parasite's lifecycle is multi-stage and complex, relying on a faecal-oral transmission route.  
73 Unsporulated, non-infective oocysts are excreted in the faeces and mature to sporulated,  
74 infective oocysts in the environment. *Eimeria* are among the most speciose eukaryotic  
75 organisms and are known to infect a wide range of vertebrate species (Ogedengbe *et al.*,  
76 2018). Pathogenic species of *Eimeria* have the potential to cause fatal coccidiosis within the  
77 host (Johnson & Reid, 1970).

78 Two species of *Eimeria*, *Eimeria crecis* and *Eimeria nenei*, have been found to parasitise the  
79 corncrake (Jeanes *et al.*, 2013). Coccidia-associated enteritis of the small intestine (which we  
80 term enteric 'coccidiosis') was diagnosed as a cause of morbidity and mortality in corncrakes  
81 reared for reintroduction from 2007 onwards (Jeanes *et al.*, 2013), although the pathogenicity  
82 of *E. crecis* and *E. nenei* – and their relative roles in the disease process – are yet to be  
83 determined (Jeanes *et al.*, 2013). Disease risk management for the reintroduction project  
84 included measures to reduce, but not preclude, coccidia infection in corncrakes being reared  
85 for release: control measures were designed to conserve the parasites within the corncrake  
86 population, to allow for maintenance of low – subclinical – levels of infection (Sainsbury, 2015).  
87 Coccidiosis control measures (in addition to the health checks and quarantine conditions at  
88 WZ and PCT), included treatment with the anticoccidial toltrazuril (Baycox 2.5% Oral  
89 Solution™, which, by 2014, was administered to chicks according to the following protocol:  
90 first, immediately prior to transport to the pre-release pens, either in drinking water at 25mg (1  
91 ml)/ L for two consecutive days, or as one dose directly per os at 7 mg/kg (0.01 ml); and then,  
92 in the pre-release pens, at 25mg/ L for two consecutive days each week) and pen  
93 management (including resting of pens, and, latterly, construction of new release pens on  
94 'clean' land). High stocking densities and stress are likely to predispose to coccidiosis  
95 outbreaks (McGill *et al.*, 2010), so these factors were minimised as far as possible during the  
96 reintroduction process.

97 Control of *Eimeria*-associated coccidiosis is essential in modern livestock production,  
98 especially within intensive farming systems such as the poultry industry (Shirley *et al.*, 2007).

99 Effective integrated control includes a requirement for sensitive and specific diagnostics, but  
100 for *Eimeria*, species identification based upon oocyst morphology alone can be challenging  
101 (Kumar *et al.*, 2014). In response, polymerase chain reaction (PCR) techniques have been  
102 developed for genus- and species-level identification that target a range of sequences within  
103 the ribosomal DNA/internal transcribed spacer repeat unit and the mitochondrial cytochrome  
104 oxidase subunit 1 (mtCOI) gene (e.g. (Schwarz *et al.*, 2009; Ogedengbe *et al.*, 2011).  
105 Equivalent tools will be of benefit to diagnosis and control of coccidiosis in captive-reared  
106 corncrakes.

107 *Eimeria* species of poultry are typically pathogenic, and their infections are short-lived in the  
108 absence of re-exposure (Blake *et al.*, 2015; Shirley *et al.*, 2007). By contrast, *Eimeria* spp.  
109 infections in cranes (Family Gruidae – also Order Gruiformes) result in additional sub-clinical,  
110 chronic extra-intestinal infections within individual hosts (Novilla *et al.*, 1989). Jeanes *et al.*  
111 (2013) detected *E. crecis* and *E. nenei* at a low level in a high proportion of wild-caught  
112 corncrakes, largely irrespective of the birds' ages, suggesting the absence of a robust  
113 protective immune response (Jeanes *et al.*, 2013). In the CRP, juvenile corncrakes in the pre-  
114 release pens were likely to be particularly susceptible to disease, since *Eimeria* sp. was known  
115 to be present in the pen environments and in the reintroduced population on the adjacent  
116 reserve, and having been raised in quarantine conditions the chicks were likely to be  
117 immunologically naive to the parasites. Also, the unavoidable stressors of transport and  
118 handling increased the likelihood of stress-induced immunosuppression and associated  
119 disease emergence at this stage of the reintroduction project (Dickens *et al.*, 2009).

120 Typically, and in poultry, *Eimeria* spp. infections are localised to the intestine (Johnson & Reid,  
121 1970). However in cranes, *Eimeria* have been found to occupy a wide range of extra-intestinal  
122 tissues (Novilla *et al.*, 1989), where they can be associated with granulomatous lesions and  
123 cause disseminated visceral coccidiosis. Novilla *et al.* (1989) proposed that the extra-  
124 intestinal lifecycle and apparent limited immunogenicity exhibited by *Eimeria* species of cranes  
125 are means by which the parasite can overcome the migratory lifestyle of the host and persist

126 within populations as they range across large areas. *Eimeria* species which infect corncrakes  
127 are genetically most closely related to those which infect crane species (Jeanes *et al.*, 2013),  
128 but it is not known whether *E. crecis* and *E. nenei* occupy sites external to the intestine within  
129 the corncrake host.

130 The aims of this study were to further understand the nature of *E. crecis* and *E. nenei* infections  
131 in captive corncrakes, including their relative pathogenicity, and to develop a novel diagnostic  
132 test for their detection. Through this study we aimed to increase understanding of the parasite  
133 genus, *Eimeria*, and for the results to inform coccidiosis management strategies for the CRP.

134

## 135 **Materials and methods**

136 ***Ethical review*** The population of corncrakes investigated during this study was considered  
137 endangered and formed part of a reintroduction program, as such ethical review was  
138 conducted and approved by ZSL prior to this study. Ethical implications were minimal, though,  
139 since samples were taken from tissue archives that had been collected during routine *post*  
140 *mortem* examinations (PMEs) conducted for the CRP.

141

## 142 ***Pathological investigation and tissue sampling***

143 ***Post mortem procedure and histological examination*** Tissue samples were subsampled  
144 from archived material that had been collected during routine PMEs of juvenile captive-bred  
145 corncrakes conducted from 2007-2014. These birds had either been euthanised at their pre-  
146 release health check due to ill-health, or found dead in their pre-release pen. PMEs had  
147 followed standard procedure (Latimer & Rakich, 1994): during each PME, a range of tissues  
148 had been sampled aseptically and tissue samples from each organ had been placed  
149 separately in individual sterile 7ml bijoux (Sigma-Aldrich, Gillingham, UK) and then stored at -  
150 80°C, or -20°C and then later -80°C.

151 In a subset of cases, and depending on the state of carcass preservation, tissue samples had  
152 also been placed in 10% formalin and submitted for histological examination: any lesions, and  
153 also sections of organs such as (proximal and/or mid) small intestine, liver and spleen (where  
154 available) had been sampled. Routine histological methods had been employed (Bancroft,  
155 2008), and 5-µm-thick sections had been examined using stains such as H&E, Ziehl-Neelsen,  
156 Giemsa, Periodic Acid-Schiff and Gram-Twort.

157

158 **Case selection and sub-sampling of tissues** Frozen, archived intestinal tissue (duodenum  
159 or jejunum) was selected from a range of individual cases, including: cases in which  
160 histological evidence of intestinal coccidiosis had been observed; cases in which coccidia  
161 infection had been diagnosed histologically but associated disease had not been observed;  
162 cases in which coccidia infection had not been detected on histological examination; and  
163 cases in which histology had not been performed on intestinal tissue. A histological diagnosis  
164 of coccidiosis had been ascribed where leukocytes – mononuclear cells and/or granulocytes  
165 – had been visualised in the lamina propria of the intestine, concurrent with coccidia parasites  
166 (oocysts and/or microgametes and/or macrogametes) in enterocytes. In some cases where  
167 histology had been performed, tissue autolysis had limited interpretation. In cases where  
168 intestinal coccidiosis had been confirmed through histological examination, liver and spleen  
169 were also sampled, where available.

170 The archived tissues were minimally defrosted and each tissue was sub-sampled in a sterile  
171 petri dish using sterile scalpel blades. For small-intestinal tissue, the area of the intestine that  
172 grossly appeared most inflamed was selected, to increase the likelihood of detecting *Eimeria*  
173 sp.. Each sub-sample of tissue was placed in a sterile 7ml bijoux (Sigma-Aldrich, Gillingham,  
174 UK) and stored at -80°C prior to molecular testing.

175

176 **Molecular diagnostic investigation**



177 **Genomic DNA isolation from tissue samples** Total genomic DNA (gDNA) was extracted  
178 from each frozen tissue using a DNeasy Blood & Tissue Kit (Qiagen, Crawley, UK) and quality  
179 controlled using a Nanodrop ND-1000 Spectrophotometer (DNA concentration >50ng/μl,  
180 260:280 ratio close to 2.0; Thermo Scientific, Basingstoke, UK) according to the respective  
181 manufacturer's guidelines.

182

183 ***Eimeria* genus-specific PCR amplification** Polymerase chain reaction was carried out  
184 targeting the 18S rDNA using the primers ERIB1 and ERIB10 as described elsewhere  
185 (Schwarz *et al.*, 2009). Briefly, all PCRs were carried out using the following reagents to make  
186 a 25μl reaction: 12.5 μl 2x MyTaq mix (Bioline, London, UK); 1μl sample DNA; 0.4μM forward  
187 and reverse primers and molecular grade water (Sigma-Aldrich, Gillingham, UK). Positive  
188 controls consisted of purified *Eimeria tenella* genomic DNA, negative controls consisted of  
189 molecular grade water (Sigma-Aldrich, Gillingham, UK). PCR products were resolved by  
190 electrophoresis using 1% (w/v) ultrapure agarose in 1% (v/v) Tris Borate EDTA buffer with  
191 0.01% (v/v) SafeView DNA stain (Invitrogen, Paisley, UK and NBS Biologicals, Huntingdon,  
192 UK). Gels were run at 50 volts and visualised under ultra violet light using a Syngene U:Genius  
193 gel imaging system (Syngene, Cambridge, UK). Amplicons from successful reactions were  
194 purified using a QIAquick PCR Purification Kit (Qiagen, Crawley, UK) and Sanger sequenced  
195 (GATC Biotech, Constanz, Germany) using the same primers employed in their original  
196 amplification. Amplicon identity was confirmed by similarity to published sequences for *E.*  
197 *crecis* (GenBank accession numbers: HE653904 and HE653905) and *E. nenei* (HE653906)  
198 using BLASTn (NCBI, Bethesda, USA) through CLC Main Workbench version 5.7.1 (CLC Bio,  
199 Prismet, Denmark).

200

201 **GeneScan primer design** The 18S rDNA sequence for *E. crecis* was compared to the chicken  
202 (*Gallus gallus*) genome (Ensembl, release 80) using BLASTn (NCBI, Bethesda, USA) which  
203 revealed considerable similarity from base pair 304 onwards (80.4% sequence coverage, E

204 value  $4.9e^{-91}$ ), indicating a possible risk of cross reaction to host DNA. Subsequent alignment  
205 of reference and 18S rDNA sequences generated in this study representing *Eimeria* which  
206 infect corncrake revealed the presence of a TTA three base pair indel (insertion or deletion)  
207 with potential for GeneScan marker development. For *E. crecis* TTA n = 2 (alignment 169-  
208 172bp). For *E. nenei* TTA n = 1 (alignment 169-171). The forward primer ERIBnf (5'-  
209 TGTCTCAAAGATTAAGCCATGC-3') and reverse primer ERIBnr (5'-  
210 CGAAGTGGGTTGGTTTTGTATC'-3) were designed using the Sigma Aldrich Oligos &  
211 Peptides Design website (Sigma-Aldrich, Gillingham, UK), incorporating a 6-  
212 carboxyfluorescein (6-FAM) modification. PCR conditions were 1 cycle 94°C for 60s, 35 cycles  
213 94°C for 30s, 57°C for 30s and 72°C for 60s, followed by 1 cycle 72°C for 10 mins.

214

215 **GeneScan PCR resolution** PCR products obtained using the ERIBnf/ERIBnr primer pair  
216 were resolved using an ABI3100 series system (Applied Biosystems/Life Technologies, UK)  
217 with the GeneScan™-ROX 500 size standard as recommended by the manufacturer. Raw  
218 data (.fsa files) were visualised using Peak Scanner 2 (Applied Biosystems). GeneScan  
219 output results were manually scored for *E. crecis* and/or *E. nenei* presence by product size  
220 (180 bp corresponded to *E. crecis*, 177 bp for *E. nenei*).

221

## 222 **Statistical analyses**

223 Statistical analyses were carried out using R (version 3.2.1) statistical software. The Chi-  
224 squared test was used to compare the proportion of birds in which each *Eimeria* species was  
225 detected. Fisher's exact test was used to compare the proportion of cases in which coccidia  
226 infection (including putative coccidia-associated enteritis) had been diagnosed through (i)  
227 histological examination, relative to (ii) molecular analysis. Similarly, the relative occurrence  
228 of single versus multi-species detection was compared (through Fishers exact test) between  
229 birds with histological evidence of intestinal coccidiosis and those in which the small intestine

230 had been examined histologically but no coccidia-related disease had been detected. P values  
231  $\leq 0.05$  were considered statistically significant.

232

## 233 **Results**

### 234 ***Pathological investigation and tissue sampling***

235 Archives presented a total of 65 birds from which tissues were available for DNA extraction  
236 (Table 1).

237

### 238 ***Molecular diagnostic investigation***

239 ***Genomic DNA isolation from tissue samples*** DNA was successfully extracted from tissues  
240 from 56 of the 65 cases (Table 1).

241 Of these 56 cases, previous histological examination had been performed in 40 cases:  
242 coccidia infection had been detected in 23 cases, and histological lesions consistent with  
243 intestinal coccidiosis had been observed in 14 of these cases (Table 1).

244

245 ***Eimeria genus-specific PCR amplification*** PCR amplification targeting the *Eimeria* genus  
246 18S rDNA locus was successful in all 56 cases (Table 2). *Eimeria* sp. DNA was detected by  
247 direct amplicon sequencing followed by BLASTn in samples from 54 (96%) of these 56 cases.  
248 No sequences different to the published references (accession numbers HE653904-6) were  
249 detected, with new flanking sequences confirmed for *E. crecis* and *E. nenei* respectively  
250 (accession numbers LT970833-4). Manual sequence curation was required to identify both  
251 *E. crecis* and *E. nenei* sequences: 45 (80% of all tested) cases were positive for *E. crecis*, and  
252 20 (36%) were positive for *E. nenei*. *E. crecis* and *E. nenei* were detected concurrently in 11  
253 (20% of all tested) cases, and trace data was used to determine the majority sequence type

254 (Figure 1): *E. crecis* was called as the dominant species in 8 cases. Minority sub-populations  
255 are unlikely to have been detected in the absence of a cloning/multiple clone sequencing  
256 protocol.

257

258 **GeneScan PCR resolution** Application of the new GeneScan assay successfully identified  
259 the presence of *Eimeria* sp. gDNA in 54 of 56 cases and allowed species-specific detection  
260 of *E. crecis* and *E. nenei* (Figure 2). *E. crecis* was detected in 45 (80%), and *E. nenei* in 24  
261 (43%), of all tested cases. *E. crecis* was significantly the most common species detected  
262 ( $\chi^2=16.65$ ;  $p<0.01$ ). These results were equivalent to the standard 18S rDNA PCR plus  
263 amplicon sequencing (Table 2), with the exception that the GeneScan assay identified four  
264 additional cases of *E. nenei* occurrence.

265

#### 266 **Molecular detection of *Eimeria* sp. relative to pathological findings**

267 Our GeneScan assay targeting the TTA indel detected *Eimeria* sp. in tissues from 39 of the  
268 40 birds in which histology had been performed – a significantly greater proportion than the  
269 23 cases in which coccidia infection had been detected histologically ( $P=0.0076$ ); only one of  
270 the cases remained negative, as it had been on histology.

271 Of the 14 cases in which intestinal coccidiosis had been diagnosed through histological  
272 examination – in which *E. crecis* was detected in the intestine of all cases, and *E. nenei* in the  
273 intestine of six cases using the GeneScan assay – liver tissue was available for all and splenic  
274 tissue was available for two cases (Table 3). *Eimeria* sp. DNA was detected using the  
275 GeneScan PCR in all of these extra-intestinal tissue samples. In each case, the *Eimeria*  
276 species detected in the liver +/- spleen was/were also detected in the intestine. *E. crecis* DNA  
277 was detected in the liver of 13 cases (i.e. there was one case in which it was detected in the  
278 intestine but not the liver), and in the spleen of both cases in which it was available. By  
279 contrast, of the six intestinal coccidiosis cases in which the intestine was *E. nenei*-positive, *E.*

280 *nenei* DNA was only detected in the liver tissue in two cases, and not in either splenic sample  
281 (both *Eimeria* species were detected in the intestine of each of these the cases). In 6 of the  
282 14 coccidiosis cases, both *E. crecis* and *E. nenei* were detected concurrently in the intestine,  
283 however there was only one case in which they were detected concurrently in liver.

284 There was found to be no significant difference in the species of *Eimeria* present between  
285 birds that had been diagnosed with coccidia infection on histology, but in which coccidiosis  
286 had not been observed (n=9), versus those diagnosed with coccidiosis (n=14) (P value=0.78).  
287 Similarly, the proportion of cases in which there was co-detection of both *Eimeria* species did  
288 not differ significantly (P value=0.18) between these two groups.

289 Of the 13 coccidiosis cases where *Eimeria* sp. DNA was detected in liver tissue, PME results  
290 reported a grossly enlarged appearance for the liver +/- 'congested' in 11 cases, and in one  
291 of these cases there had also been multiple small firm white foci over the liver surface,  
292 however, these findings were considered a potential artefact of barbiturate euthanasia +/-  
293 another concurrent disease. The liver had been examined histologically in 12 of these cases,  
294 and observations had included changes consistent with: haematopoiesis (6 cases),  
295 intracellular lipid +/- glycogen deposition (4 cases), autolysis (2 cases), haemosiderosis (2  
296 cases), euthanasia artefact (1 case), and, in one case (in which no gross abnormalities of the  
297 liver had been noted), mild, necrotising, multi-focal inflammation. Of the 2 coccidiosis cases  
298 in which spleen tested *Eimeria* sp.-positive, at PME the spleen had grossly appeared enlarged  
299 or 'congested', which (again) was a potential artefact of barbiturate euthanasia +/- another  
300 concurrent disease. The spleen had been examined histologically in both cases and no  
301 abnormalities had been observed.

302

## 303 **Discussion**

304 *Eimeria*-derived coccidiosis in the corncrake was highlighted as a concern for the CRP  
305 following the death of a number of juvenile birds from coccidiosis in pre-release pens

306 (Sainsbury, 2015). The approaches adopted to rear and release corncrake bear some  
307 similarities to gamebirds such as pheasants, partridge and bobwhite quail, where coccidiosis  
308 has become a notable health issue (i.e. Gerhold *et al.*, 2016; Liou *et al.*, 2001; Ruff *et al.*,  
309 1987); although corncrake are more closely related to other gruiformes, such as the cranes.

310 This study identified *E. crecis* and *E. nenei* in juvenile (approximately 2-5 week old) corncrakes  
311 which had died in the pre-release phase of a reintroduction project. *Eimeria* sp. DNA was  
312 detected in tissues from 54 of 56 birds tested. While not absolute, we considered positive  
313 DNA detection to be indicative of current or recent infection with viable parasites, and therefore  
314 considered the results to be consistent with a very high infection prevalence in this population  
315 of birds submitted for PME. All PCR reactions were accompanied by negative controls,  
316 indicating the absence of laboratory contamination. The level of occurrence was consistent  
317 with previous studies based on faecal oocyst excretion from wild corncrake (Jeanes *et al.*,  
318 2013). Microscopic detection of coccidial organisms during histology remains the Gold  
319 Standard measure of parasite occurrence, but it should be noted that such evidence was only  
320 available here for the samples from the intestine. It is possible that parasite numbers in the  
321 liver and spleen would have been lower than the intestine for these naturally infected birds,  
322 undermining attempts at microscopic detection. High-level experimental parasite challenge  
323 could resolve the question, although such an approach was not possible with these captive  
324 birds. *E. crecis* appeared to be the more common *Eimeria* species in this corncrake  
325 population. The high level of occurrence precluded detection of an association between  
326 infection status and the presence of coccidia-associated disease, and indicated that whilst  
327 both corncrake *Eimeria* species may have the potential to be pathogenic, their infections  
328 probably follow a chronic course, and that the onset of coccidiosis is likely to be triggered by  
329 extraneous factors such as stress or high-level oocyst challenge (environmental  
330 contamination). Quantitative PCR could be used in future to investigate whether these *Eimeria*  
331 sp. have a dose-related pathological effect in the corncrake.

332 A high prevalence of *Eimeria* sp. infection was not surprising to the authors, because we  
333 considered that most, if not all, juvenile birds were likely to have been exposed to coccidia  
334 oocysts in their pre-release pens, and because (as per above) a previous study found an  
335 infection prevalence of up to 86% in wild corncrakes (Jeanes *et al.*, 2013). Samples in this  
336 current study therefore showed an even higher prevalence than for wild corncrakes, possibly  
337 because of a higher rate of environmental challenge, but also because *post mortem* intestinal  
338 samples are likely to be a more sensitive diagnostic tool than single faecal samples (used by  
339 Jeanes *et al.*, 2013), since oocyst shedding in faeces can vary temporally (Villanúa *et al.*,  
340 2006). Also, tissue samples for this study were taken from birds which had died pre-release  
341 – the prevalence of infection in birds that appeared healthy and were released might well have  
342 been lower than in those birds submitted for PME.

343 The presence of *E. crecis* and *E. nenei* DNA in the liver and spleen of corncrakes is a novel  
344 finding, however closely-related *Eimeria* species of cranes typically infect extra-intestinal  
345 tissues – including the liver and/or spleen – where they can elicit granulomatous lesions and  
346 cause disseminated visceral coccidiosis; this disease is most common in young cranes  
347 (Carpenter *et al.*, 1992; Novilla & Carpenter, 2004). Natural extra-intestinal coccidia infections  
348 have also been observed in some other avian and mammalian species such as *E. truncata*  
349 and *E. stiedae*, in the goose and rabbit respectively (Long, 1970; Novilla & Carpenter, 2004;  
350 Ball *et al.*, 2014). Of our corncrake cases in which *Eimeria* DNA was detected in liver +/-  
351 spleen, *Eimeria* parasites had not been visible histologically in those tissues, neither had there  
352 been gross or histological evidence of hepatic or splenic inflammation, with the exception of  
353 one case, which had had very mild, multi-focal hepatic necrosis. Therefore, we were unable  
354 to determine the pathogenicity of extra-intestinal *Eimeria* sp. infection in corncrakes.

355 Our findings support growing evidence that extra-intestinal infection forms a mechanism to  
356 overcome the migratory lifecycle of these gruiform hosts (Jeanes *et al.*, 2013). In order to  
357 investigate this aspect further, liver and spleen samples could also be analysed from birds  
358 which showed no sign of coccidiosis upon PME. Detection of the parasite in these organs

359 would add further support for an altered, non-limited parasite lifecycle similar to that of crane-  
360 adapted *Eimeria* species. Analysis of other tissue samples, for example lung and kidney,  
361 could further identify the extent of the infection and serve as another comparison to *Eimeria*  
362 sp. infections of cranes (Novilla & Carpenter, 2004).

363 *Eimeria* species are characteristically highly host and site-specific (Shirley *et al.*, 2007),  
364 however as of yet nothing is known about site specificity in *E. crecis* and *E. nenei* within the  
365 corncrake intestine, or whether one species is more adept at extra-intestinal migration. The  
366 higher parasite prevalence demonstrated through PCR as opposed to histology demonstrated  
367 that infection in some birds was probably missed on histological examination of intestinal  
368 tissue, due to e.g. low-level infection, tissue autolysis, and possibly because, generally, only  
369 one or two finite sections of small intestine had been sampled for histological examination at  
370 PME. Changes to PME protocol have since been instigated, to ensure that multiple sections  
371 of the intestinal tract are sampled in a standardised manner, which will, for example, allow  
372 future work to investigate whether *E. crecis* and *E. nenei* have a predilection to particular areas  
373 of the intestinal tract, as in poultry (Johnson & Reid, 1970). In cases of intestinal coccidiosis,  
374 when *E. crecis* was found in the intestine, its DNA was also detected in extra-intestinal tissue  
375 (with one exception), whereas *E. nenei* was rarely detected outside the intestine, indicating  
376 potentially more limited systemic spread than for *E. crecis*: we hypothesise that *E. crecis* may  
377 be more adept at extra-intestinal migration. Alternatively, however, this could have been an  
378 artefact of our relatively small sample size – given that we were working with a species of high  
379 conservation concern. Further investigation of both of these points would greatly benefit  
380 understanding of these parasite species.

381 A species-specific set of primers were designed for a GeneScan-style analysis, and provided  
382 slightly increased sensitivity as a diagnostic tool to identify the two species of *Eimeria* when  
383 compared to primers previously available for standard PCR and amplicon sequencing, or  
384 through detection by histopathology. A possible explanation for the increased sensitivity of  
385 these primers is that sequencing results from coinfections using the 18S rDNA primer pair rely



386 upon sufficient DNA from both species being present to be detected on overlying traces. By  
387 contrast, using the GeneScan primers to detect the TTA indel may be more sensitive at  
388 detecting low levels of infection, benefitting from a smaller amplicon and possible greater PCR  
389 efficiency.

390 Future uses of the GeneScan PCR primers as a diagnostic tool could include analysis of faecal  
391 samples; PCR methods for detection of *Eimeria* sp. in faecal samples have recently been  
392 developed for health monitoring of endangered whooping cranes (*Grus Americana*) (Bertram  
393 *et al.*, 2015). In the context of the CRP, this could, first, confirm the point at which chicks are  
394 exposed to the parasite during rearing; and, second, enable the prevalence of infection in  
395 juvenile birds that are ultimately released to be compared to the prevalence in individuals  
396 submitted for PME. Future diagnostics for *Eimeria* which infect corncrakes would benefit from  
397 access to additional sequence markers. While the GeneScan assay presented here worked  
398 well in samples collected in Southeast England, proving consistent with results from the  
399 Hebrides (Jeanes *et al.*, 2013), it is noted that an indel marker may be influenced by  
400 expansion/contraction which could result in unreliable results. Further sequencing from a more  
401 diverse panel of isolates will be required to assess stability, while the development of markers  
402 such as mtCOI would be appropriate (Ogedengbe *et al.*, 2011).

403 Disease management practices, including toltrazuril treatment, broadly appear to have  
404 successfully suppressed the occurrence of coccidiosis in corncrakes reared for the CRP and  
405 to have maintained infection at a predominantly sub-clinical level. A prophylactic, in-feed  
406 treatment regime with an ionophore (coccidiostat) might promote better development of  
407 natural immunity to coccidia infection and could be considered preferable to our current  
408 toltrazuril-based strategy (Carpenter *et al.*, 1992; Blake *et al.*, 2017); however, for a number  
409 of reasons, toltrazuril treatment is more practicable in the case of the CRP. An alternative  
410 control strategy that could be investigated, for which this current study formed a useful piece  
411 of background research, would be vaccination of chicks with a controlled level of *Eimeria* sp.  
412 oocysts (McDonald & Shirley, 2009; Sharman *et al.*, 2010). Allowing juvenile corncrakes to

413 encounter *Eimeria* sp. during the early-rearing stage may encourage early development of  
414 immunity and better enable them to acclimatise to oocyst burdens encountered in the pre-  
415 release and release environments. Vaccination would initially need to be highly controlled,  
416 however, to identify any pathogenic effects and to determine the suitable timing and dosage  
417 of oocysts. Nonetheless, such a strategy has been proposed for use with captive reared  
418 pheasants (Liou *et al.*, 2001).

419 In conclusion, our results provide evidence for a high prevalence of *E. crecis* and *E. nenei*  
420 infection in juvenile captive-reared corncrakes presented for PME. We present a GeneScan  
421 assay that detects *E. crecis* and *E. nenei* with greater sensitivity than those primers previously  
422 available for standard PCR. The presence of both species of *Eimeria* in intestinal samples  
423 showed no significant relation to disease status, suggesting that neither species should be  
424 considered highly pathogenic. The novel finding of *E. crecis* and *E. nenei* DNA in liver and  
425 spleen provides support for an extra-intestinal life cycle, similar to that seen in *Eimeria* species  
426 infecting cranes (Novilla & Carpenter, 2004). This finding further supports a migratory  
427 hypothesis for *Eimeria* sp. evolution in Gruiformes, i.e. that the parasite's life cycle has evolved  
428 as an adaptation to the host species' migratory behaviour (Novilla & Carpenter, 2004).

429

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439

440 **Disclosure statement**

441 The authors of this paper disclose that no financial interest or benefit will arise from the direct  
442 applications of their research.

443

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538 check table formatting inc superscript and use of vertical lines for all... (Table 3)

539

540 **Table 1.** Number of cases from which tissue samples were available and in which DNA extraction was successful, and histological findings

	Total	Histological examination			
		No	Yes.		
		Histological appearance of proximal and/or mid intestine:			
		No coccidia infection detected	Coccidia observed, enteritis not detected	Confirmed coccidiosis (coccidia-associated enteritis)	
gDNA extraction attempted	65	24	18	9	14
Extraction unsuccessful	9	8	1	0	0
PCR testing performed	56	16	17	9	14

541

542



543 **Table 2.** Number of birds positive for *Eimeria* spp. using: 18S rRNA gene primers (ERIB1 and ERIB10) coupled with direct amplicon sequencing;  
 544 and TTA indel primers (ERIBnf and ERIBnr) coupled with GeneScan resolution

Target DNA region	No. cases tested	No. ( <i>and percentage</i> ) of tested cases positive for <i>Eimeria</i> sp.			
		<i>Eimeria</i> sp.	<i>Eimeria crecis</i>	<i>Eimeria nenei</i>	<i>E. crecia</i> & <i>E. nenei</i> (concurrent detection)
18S	56	54 (96%)	45 (80%)	20 (36%)	11 (20%)
TTA indel	56	54 (96%)	45 (80%)	24 (43%)	15 (27%)

545

546

547 **Table 3.** Number of cases positive for *Eimeria* sp. DNA (and total number of cases tested) using the GeneScan assay, and associated  
 548 histological findings. The number of cases from which each particular *Eimeria* species was detected is indicated on the second line of each row  
 549 as follows: *E. crecis* only / *E. nenei* only / concurrent detection of both *Eimeria* species

Tissue samples available	Histology?				Total cases
	No	Yes.			
		Histological appearance of proximal and/or mid intestine:			
	No coccidia infection detected	Coccidia observed, enteritis not detected	Confirmed coccidiosis (coccidia-associated enteritis)		
Intestine only	15 (16)	16 (17)	9 (9)	NA	40 (42)
	7 / 6 / 2	9 / 2 / 5	6 / 1 / 2		22 / 9 / 9
Intestine and liver	NA	NA	NA	12 (12) <sup>a</sup>	12 (12)
				8 / 0 / 4	8 / 0 / 4
Intestine, liver & spleen	NA	NA	NA	2 (2) <sup>a</sup>	2 (2)
				0 / 0 / 2	0 / 0 / 2
Total cases	15 (16)	16 (17)	9 (9)	14 (14) <sup>a</sup>	54 (56)
	7 / 6 / 2	9 / 2 / 5	6 / 1 / 2	8 / 0 / 6	30 / 9 / 15

550 <sup>a</sup>In each case where multiple tissue types were tested, all of the tissues were positive for *Eimeria* sp.. *E. crecis* and *E. nenei* were detected  
 551 concurrently in the intestine in six cases, and concurrently in the liver in one of these cases.

552

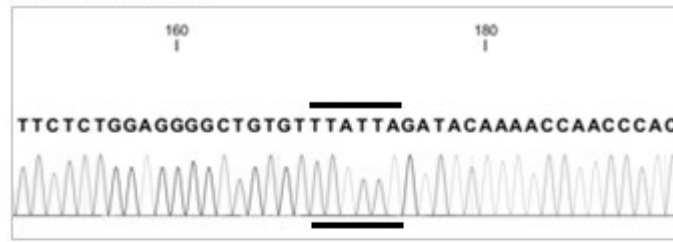
553 **Figure legends**

554 **Figure 1.** Sequence traces viewed using CLC Main Workbench version 5.7.1 (CLC Bio,  
555 Prismet, Denmark). The TTA indel is highlighted by a bold line above and below the trace. (a)  
556 The *E. crecis* trace includes the TTA repeat. (b) The *E. nenei* trace has no TTA repeat. (c)  
557 Both *E. crecis* & *E. nenei* sequences are present, with *E. crecis* identified semi-quantitatively  
558 as the majority species on the trace as shown by the TTA repeat, however the *E. nenei*  
559 sequence was also visible at lower level.

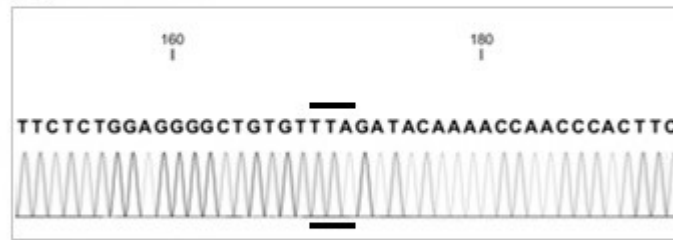
560

561 **Figure 2.** Excised sections of GeneScan outputs from Peak Scanner 2. (a) Results obtained  
562 from samples previously shown to have no eimerian DNA present. (b) Samples containing *E.*  
563 *crecis*, as shown by a blue arrow at 180 bp peak. (c) Samples containing *E. nenei*, as shown  
564 by a green arrow at 177 bp peak. (d) Samples containing both species as shown by blue and  
565 green arrows. Non-target peaks (red) represent marker samples of known molecular length  
566 as standards. RFU = relative fluorescence units.

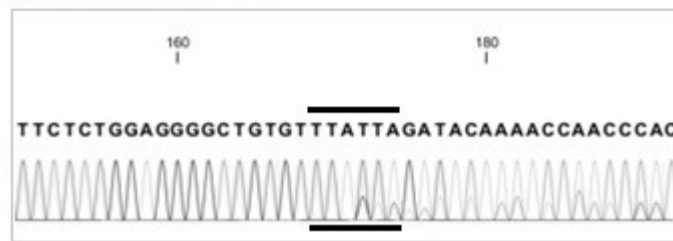
(a) *Eimeria crecis*

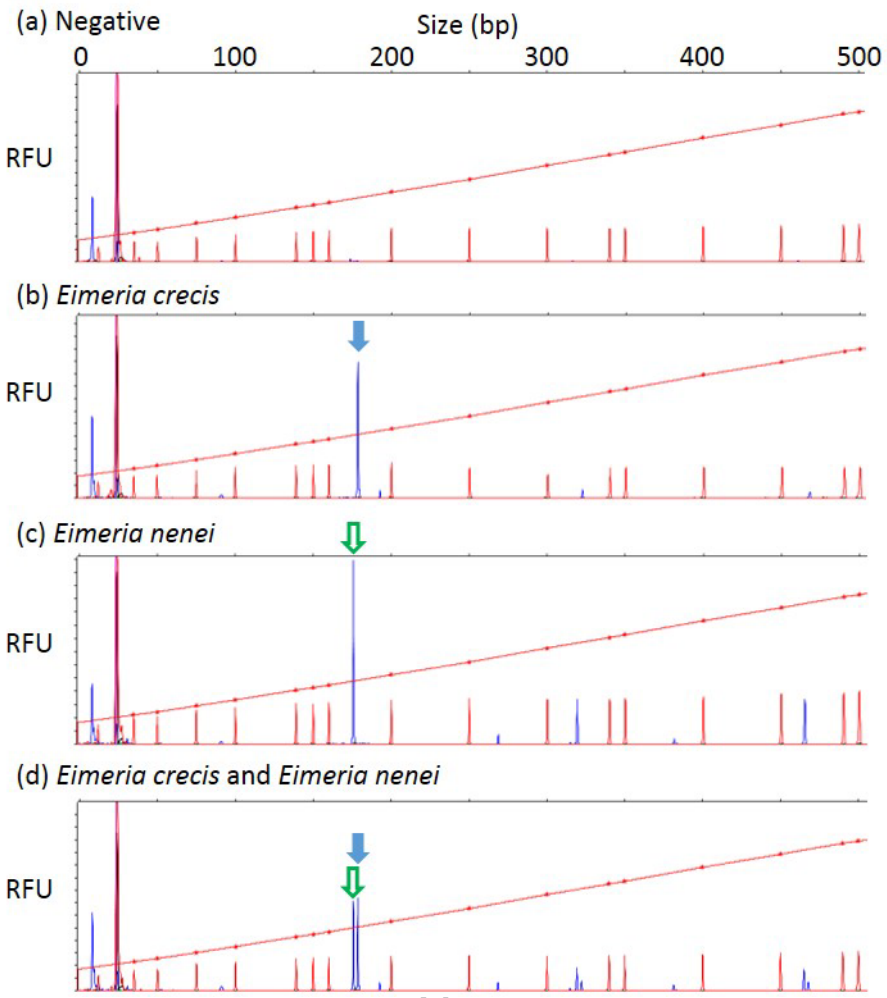


(b) *Eimeria nenei*



(c) *E. crecis* & *E. nenei*





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