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TITLE

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

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ABSTRACT

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

Research highlights

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

Introduction

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

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

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

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

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

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

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

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

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

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

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

Materials and methods

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

Pathological investigation and tissue sampling

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

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

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

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

Molecular diagnostic investigation

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

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

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

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

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

Statistical analyses

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

had been examined histologically but no coccidia-related disease had been detected. P values ≤ 0.05 were considered statistically significant.

Results

Pathological investigation and tissue sampling

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

Molecular diagnostic investigation

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

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

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

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

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

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

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

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

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

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

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

Discussion

Eimeria-derived coccidiosis in the corncrake was highlighted as a concern for the CRP 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.

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

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

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

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

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

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

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

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

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

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

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

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Disclosure statement

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

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

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

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. (*and percentage*) of tested cases positive for *Eimeria* sp.			
		Eimeria sp.	*Eimeria crecis*	*Eimeria nenei*	*E. crecia* & *E. nenei* (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*) 7 / 6 / 2	16 (*17*) 9 / 2 / 5	9 (*9*) 6 / 1 / 2	NA	40 (*42*) 22 / 9 / 9
Intestine and liver	NA	NA	NA	12 (*12*)[a] 8 / 0 / 4	12 (*12*) 8 / 0 / 4
Intestine, liver & spleen	NA	NA	NA	2 (*2*)[a] 0 / 0 / 2	2 (*2*) 0 / 0 / 2
Total cases	15 (*16*) 7 / 6 / 2	16 (*17*) 9 / 2 / 5	9 (*9*) 6 / 1 / 2	14 (*14*)[a] 8 / 0 / 6	54 (*56*) 30 / 9 / 15

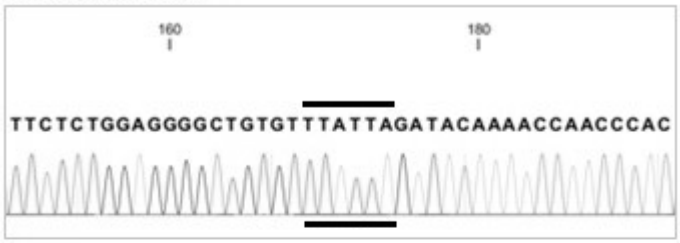
550 [a]In each case where multiple tissue types were tested, all of the tissues were positive for *Eimeria* sp.. *E. crecis* and *E. nenei* were detected
551 concurrently in the intestine in six cases, and concurrently in the liver in one of these cases.
552

Figure legends

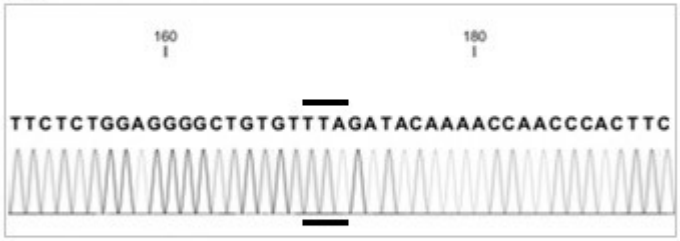
Figure 1. Sequence traces viewed using CLC Main Workbench version 5.7.1 (CLC Bio, Prismet, Denmark). The TTA indel is highlighted by a bold line above and below the trace. (a) The *E. crecis* trace includes the TTA repeat. (b) The *E. nenei* trace has no TTA repeat. (c) Both *E. crecis* & *E. nenei*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

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

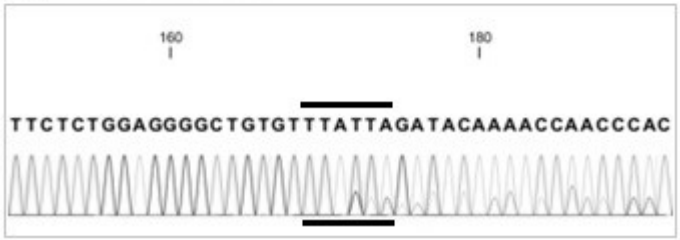
(a) *Eimeria crecis*



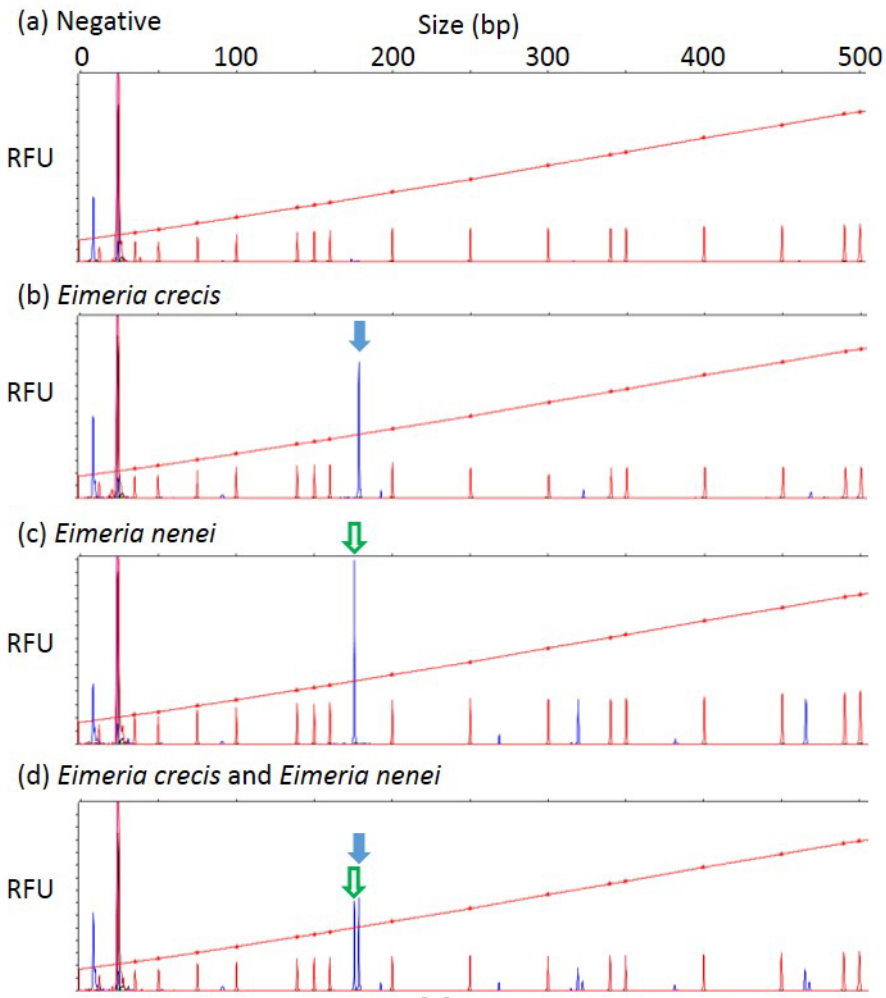
(b) *Eimeria nenei*



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



575



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