Eimeria spp. infection in captive-reared corncrakes (*Crex crex*): application of a Genescan diagnostic indicates high prevalence of infection and extra-intestinal life stages

Journal:	*Avian Pathology*
Manuscript ID	Draft
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Serna, Hannah; Institute of Zoology of the Zoological Society of London Pocknell, Ann; Finn Pathologists Sainsbury, Tony; Institute of Zoology of the Zoological Society of London Peniche, Gabriela; Institute of Zoology of the Zoological Society of London Blake, Damer; Royal Veterinary College, Pathology and Pathogen Biology Beckmann, Katie; Institute of Zoology of the Zoological Society of London,
Keywords:	Rallidae, Wild bird, Coccidiosis, Corncrake Crex crex, Eimeria sp.

TITLE

Eimeria spp. infection in captive-reared corncrakes (*Crex crex*): application of a Genescan diagnostic indicates high prevalence of infection and extra-intestinal life stages

AUTHORS

Hannah Serna[1, 2], Ann Pocknell[3], Anthony W. Sainsbury[2], Gabriela Peniche[2], Damer P. Blake[4] and Katie M. Beckmann[2]*

AFFILIATIONS

[1]The Royal Veterinary College, 4 Royal College St, London, NW1 0TU; [2]Institute of Zoology, Zoological Society of London, Regent's Park, London, NW1 4RY; [3]Finn Pathologists, The Old Schoolhouse, Hoxne Road, Weybread, Suffolk, IP21 5TT; [4]Department of Pathobiology and Population Sciences, The Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Herts, UK, AL9 7TA

*CORRESPONDING AUTHOR

Katie M. Beckmann katie.beckmann@ioz.ac.uk

ABSTRACT

Eimeria crecis and *E. nenei* have been detected in association with enteric disease ('coccidiosis') in the corncrake (*Crex crex:* Family Rallidae, Order Gruiformes). Both parasite species are known to cycle persistently in apparently healthy free-living corncrakes, but captive-bred juvenile birds reared for a corncrake reintroduction project in the UK appeared particularly susceptible to clinical disease. Here, we investigated the occurrence of *E. crecis* and *E. nenei* in juvenile corncrakes and developed a species-specific polymerase chain reaction (PCR) as a diagnostic tool for their identification. PCR amplification and sequencing of 18S rDNA was performed using genomic DNA extracted from corncrake intestine, liver and spleen samples. A high prevalence of infection was revealed, including detection in liver and spleen tissue. Extra-intestinal detection has previously been described for closely-related *Eimeria* species in cranes (Family Gruidae, Order Gruiformes) and suggested as an evolutionary adaptation to the host's migratory nature. *Eimeria crecis* was found to be significantly more common than *E. nenei* in this corncrake population. No association was found between infection status (presence/absence) and the occurrence of coccidia-associated disease, precluding determination of the *Eimeria* species' relative pathogenicity. Sequences generated were used to design a GeneScan diagnostic PCR targeting a species-specific TTA indel located within the 18S rDNA. Application of the GeneScan assay suggested greater sensitivity than 18S rDNA/amplicon sequencing approach. Artificial exposure of captive-bred juvenile corncrakes to *E. crecis* and *E. nenei* could be investigated as a potential management option, to confer immunity prior to higher-level environmental exposure later in the translocation process.

Research highlights

- High prevalence of *Eimeria* spp. infection (*E. crecis* significantly more common)
- Detection of *E. crecis* and *E. nenei* 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 contribute to a more intensive grassland management system (Green *et al.*, 1997). Following conservation efforts initiated in 1992, populations in the Scottish 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 southern England through development of a viable breeding population in East Anglia (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 and reared to 10-14 days of age in quarantine facilities at ZSL Whipsnade Zoo (WZ) and PCT, and then transferred to pre-release pens in the release region, where 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 (Lillehoj & Trout, 1993). 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 (Lillehoj & Trout, 1993). *Eimeria* are among the most speciose eukaryotic organisms and are known to infect a wide range of vertebrate species. Pathogenic species of *Eimeria* have the potential to cause fatal coccidiosis within the host (Lillehoj & Trout, 1993).

Two species of *Eimeria, Eimeria crecis* and *E. 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 – were not 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. Controlled parasite exposure can induce an element of immune protection prior to natural challenge following release. Control measures were therefore designed to conserve the parasites within the corncrake population and maintain low levels of infection, i.e. sub-clinical infection status, where possible, rather than to completely eliminate the parasites (Sainsbury, 2015). Coccidiosis control measures (in addition to the health checks and quarantine conditions at WZ and PCT) included a prophylactic course of treatment with an anticoccidial (toltrazuril: Baycox) for all birds in the pre-release pens, and pen management (including re-turfing and 'resting' of pens, and construction of new release pens on 'clean' land, whenever practicable). High stocking densities and stress are likely to predispose to coccidiosis outbreaks (McGill *et al.*, 2010) and 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 targeting a range of sequences within the ribosomal DNA/internal transcribed spacer repeat unit and the cytochrome oxidase subunit 1 (COI) gene (e.g. (Schwarz *et al.*, 2009; Ogedengbe *et al*., 2011). Equivalent tools will be of benefit to assess risk and support control of coccidiosis in captive-reared corncrakes.

Eimeria are often described as hit-and-run parasites: species which infect poultry are typically pathogenic and their infections are relatively short-lived (Blake *et al.*, 2015; Shirley *et al*., 2007). By contrast, previous studies of *Eimeria* spp. infections in cranes (Family Gruidae, Order Gruiformes) have suggested a greater degree of enzootic stability within the host population (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 (Jeanes *et al.*, 2013). Juvenile corncrakes arriving at the pre-release pens as part of the CRP were likely to be particularly susceptible to disease, since they had previously been raised in quarantine conditions and were therefore likely to be immunologically naive to the parasites, which were known to be present in the pre-release area and the reintroduced population on the adjacent reserve. The unavoidable stressors of transport and handling also increased the likelihood of stress-induced immunosuppression and associated disease emergence at this stage of the reintroduction project (Dickens *et al*., 2009).

Typically, as in poultry, *Eimeria* spp. infections are localised to the intestine (Shirley *et al.*, 2007). However in cranes, *Eimeria* have been found to occupy a wide range of host tissues, including the liver and the spleen (Novilla *et al*., 1989) where they can be found in association with the development of granulomas and can cause disseminated visceral coccidiosis. One potential driver for the extra-intestinal lifecycle and apparent limited immunogenicity exhibited by *Eimeria* species of cranes has been termed the migratory hypothesis (Novilla *et al*., 1989). It has been suggested that these properties are a means

by which the parasite can overcome the migratory lifestyle of the host and persist within populations as they range across large areas. *Eimeria* species which infect corncrake 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 of infection external to the intestine within the corncrake host.

The aims of this study were to understand further the pathobiology of *E. crecis* and *E. nenei* infections in captive corncrakes and to develop a novel diagnostic test to determine their relative occurrence in both breeding and juvenile birds. Through this study we aimed to increase understanding of the parasite genus, *Eimeria*, within a conservation setting, and to obtain data to inform future disease management strategies for corncrake coccidiosis.

Materials and methods

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

Association of pathobiology with coccidia occurrence

Post mortem procedure and histological examination Tissue samples used in this study were subsampled from archived material that had been collected during routine PMEs of juvenile 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. *Post mortem* examinations had followed standard procedure (Latimer & Rakich, 1994): during each PME, a range of tissues had been sampled 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 -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 there had been histological evidence of intestinal coccidiosis; cases in which coccidia infection had been diagnosed histologically in the apparent absence of associated disease; cases negative for coccidia infection on histological examination; and cases in which histology had not been performed on intestinal tissue. Additionally, liver and spleen were sampled (where available) from birds in which intestinal coccidiosis had been confirmed through histopathology.

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 sample that grossly appeared most inflamed was selected, to increase the likelihood of detecting *Eimeria* spp.. 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 of coccidia infection

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/ul, 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).

Development of a GeneScan diagnostic assay

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 previously described universal, genus specific primer, ERIB1 (Schwarz *et al*., 2009) was used as a forward primer to amplify the target region. The reverse primer ERIBn (5'-CGAAGTGGGTTGGTTTTGTATC'-3) was 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 ERIB1 and ERIBn primer pair were resolved using an ABI3100 series system (Applied Biosystems/Life Technologies, UK) with the GeneScan™ -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 infected with each *Eimeria* species. 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 infection 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

Molecular diagnostic investigation of coccidia infection

Genomic DNA isolation from tissue samples Archives presented a total of 65 birds from which tissues were available for study. A summary of the number admitted to the study, tissue type available and histopathological findings can be found in Table 1.

Eimeria genus-specific PCR amplification PCR amplification targeting the *Eimeria* genus 18S rDNA locus was carried out for all tissue samples and was successful for 56 cases (Table 1). *Eimeria* infection was confirmed by direct amplicon sequencing followed by BLASTn in 70 (97.2%) of 72 tissue samples, representing 54 (96.4%) of these 56 individuals. No sequences different to the published references (accession numbers HE653904-6) were detected. Manual sequence curation was required to identify both *E. crecis* and *E. nenei* sequences, and trace data was used to determine the majority sequence type where co-infection was detected (Figure 2). In 9 (75%) of 12 tissue samples, representing 8 of 11 birds, where a coinfection was detected, *E. crecis* was called as the dominant species.

GeneScan PCR resolution Application of the new GeneScan assay successfully identified the presence of *Eimeria* gDNA in 70 samples representing 54 birds and allowed species-specific detection of *E. crecis* and *E. nenei* (Figure 3). *Eimeria crecis* was significantly the most common species (χ^2=16.65; p<0.01), being detected in 60 samples representing 45 birds, whereas *E. nenei* was detected in 26 samples representing 24 birds.

Results for any *Eimeria* sp. and *E. crecis* were equivalent to the standard 18S rDNA PCR plus amplicon sequencing (Table 2), but the GeneScan assay identified additional cases of *E. nenei* occurrence.

Association of pathobiology with coccidia occurrence

Molecular and histological diagnoses Of the 56 cases in which molecular testing for *Eimeria* infection was performed, there were 40 cases in which intestinal (+/- other) tissue had been examined histologically (Table 1). Of these 40 cases, there had been histological evidence of coccidia infection in 23 cases, and none in 17 cases. Our molecular testing, using primers designed to target the TTA indel, detected *Eimeria* infection in tissues from 39 birds from this sample group (a significantly greater proportion than through histology, P=0.0076), with only 1 case remaining negative, as it had been on histology.

Detection in liver and spleen There were 14 cases in which intestinal coccidiosis (coccidia-associated enteritis) had been diagnosed through histological examination (all of which, as per above, were positive for *Eimeria* sp. on intestinal PCR). Liver tissue was available from all of these cases, and splenic tissue was available in two cases. *Eimeria* sp. DNA was detected by PCR in the liver +/- spleen of each of the 14 cases. The *Eimeria* species detected in the liver or spleen were the same as those found in the intestine, in all cases (Figure 4). *E. crecis* was found in the extra-intestinal tissue in 13 of the 14 cases in which it was present in the intestinal tissue (Figure 4); by contrast, *E. nenei* was only found in the liver tissue of 2 of the 6 cases where it was present in the intestine (Figure 4).

Of the 14 coccidiosis cases where liver tested *Eimeria* sp. positive, the liver had grossly appeared enlarged +/- 'congested' in 11 cases, and in one of these cases there had also been multiple small firm white foci over the liver surface. In all cases these findings were considered a potential artefact of barbiturate euthanasia +/- other 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, the spleen had grossly appeared enlarged or 'congested', which (again) was a potential artefact of barbiturate euthanasia +/- other concurrent disease. The spleen had been examined histologically in both cases and no abnormalities had been observed.

Co-infection In 6 of the 14 coccidiosis cases, there was intestinal co-infection with both *Eimeria* species, however co-infection was only detected in the liver in one of these 6 cases (and not in the spleen) (Figure 4).

Comparison of infection status There was found to be no significant difference in the species of *Eimeria* present between birds that were diagnosed (through gross PME and histological examination) with coccidia infection in the apparent absence of enteritis (n=9), versus those diagnosed with coccidiosis (n=14) (P value=0.78). Similarly, the proportion of cases in which there was coinfection with both *Eimeria* species, as opposed to infection with a single species, did not differ significantly (P value=0.175) between these two groups.

Discussion

Eimeria-derived coccidiosis in the corncrake is a disease which the CRP has, to date, managed through prophylactic drug treatment and pen management. It was highlighted as a concern for the project following the death of a number of juvenile birds from coccidiosis in pre-release pens over the years (Sainsbury, 2015).

This study identified *E. nenei* and *E. crecis* infection in juvenile (approximately 2-5 week old) corncrakes which had died whilst held in pre-release pens prior to reintroduction. *Eimeria* sp. infection was detected in 54 of 56 birds sampled in this study, indicating a very high infection prevalence in birds submitted for PME, with *E. crecis* the more common species in this population. Such high levels of infection found in this study precluded detection of an association between infection status and the presence of coccidia-associated disease at time of PME, and indicate that whilst both *Eimeria* species may have the potential to be pathogenic, the onset of coccidiosis is likely to be triggered by other factors such as stress or high-level oocyst challenge (environmental contamination). qPCR could be used in future to further investigate whether *Eimeria* sp. has a dose-related pathological effect in the corncrake.

A species-specific set of primers were designed for a GeneScan-style analysis, and provided 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 enough DNA from both species being present to be detected on overlying traces. By contrast, sequencing using the GeneScan primers over the TTA indel may be more sensitive at detecting low levels of infection, benefitting from a smaller amplicon and possible greater PCR efficiency.

A high prevalence of *Eimeria* sp. infection was not unexpected prior to this study, and fits with previous work that found an infection prevalence of up to 86% in wild corncrakes (Jeanes *et al.*, 2013). Samples in this current study, however, showed an even higher prevalence, and this high prevalence can be accounted for by a number of factors. First, *post mortem* intestinal samples are likely to be a more sensitive diagnostic tool than faecal samples (used by Jeanes *et al.*, 2013), as tissue samples do not rely on the parasite shedding oocysts in faeces to obtain positive results, i.e. fewer false-negative results would

be expected in comparison to faecal sampling (Villanúa *et al.*, 2006). *Eimeria* oocyst shedding has been well investigated in other species of bird and has been found to vary temporally (Villanúa *et al.*, 2006): this also could lead to under-estimation of prevalence through faecal sampling. Tissue samples for this study were taken from birds which had died whilst in the pre-release pens – the prevalence of infection in birds that appeared healthy, and were released, might well have been lower than in those birds submitted for PME. In order to investigate occurrence levels further, tissue samples from different age-categories of corncrake, such as chicks which had only encountered the early-stage quarantine facilities, could undergo molecular investigation using the primer pair designed in this study.

The presence of *E. crecis* and *E. nenei* in the liver and spleen of corncrakes is a novel finding, though closely-related species of *Eimeria* which infect cranes have been found in association with granulomatous lesions in the liver and other organs (Novilla & Carpenter, 2004). Though this study only detected parasite DNA in these tissues, the close relationship between corncrake and crane *Eimeria*, and the fact that the *Eimeria* species detected matched those in the intestine in each case, suggests that the finding was valid, i.e. consistent with infection with viable parasites rather than DNA transfer or sample contamination (Novilla & Carpenter, 2004). Of the cases with confirmed hepatic +/- splenic infection, there had been no gross or histological evidence of inflammatory lesions in those tissues, with the exception of one case, which had had very mild, multi-focal hepatic necrosis: whether or not those lesions were associated with *Eimeria* sp. infection could not be determined.

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 infection prevalence demonstrated through PCR as opposed to histology demonstrated that infection in some birds may have been missed during post mortem tissue

361 sampling for formalin fixation: in most cases only one or two finite sections of small intestine
362 had been fixed for histological examination. Changes to post mortem protocol have since
363 been instigated, to ensure that multiple sections of the intestinal tract are sampled in a
364 standardised manner, which will, for example, allow future work to investigate whether *E.*
365 *crecis* and *E. nenei* have a predilection to particular areas of the intestinal tract. Where *E.*
366 *crecis* was found in the intestine, it was also present in extra-intestinal tissue (with one
367 exception), whereas *E. nenei* was rarely found outside the intestine. In one case where a co-
368 infection was found in liver tissue, *E. nenei* was absent from spleen tissue, indicating
369 potentially limited extra-intestinal infection. It is hypothesised that *E. crecis* may be more
370 adept at extra-intestinal migration. Alternatively, however, this could have been an artefact
371 of our relatively small sample size – given that we were working with a species of high
372 conservation concern. Further investigation of both of these points would greatly benefit
373 understanding of these parasite species.

374 Our findings support growing evidence that extra-intestinal infection forms a mechanism to
375 overcome the migratory lifecycle of these gruiform hosts (Jeanes *et al*., 2013). In order to
376 investigate this aspect further, liver and spleen samples could be analysed from birds which
377 showed no sign of coccidiosis upon PME. Infection in these organs would add support for
378 an altered, more enzootic parasite lifecycle similar to that of crane-adapted *Eimeria* species.
379 Analysis of other tissue samples, for example lung and kidney, could further identify the
380 extent of the infection and serve as a further comparison to *Eimeria* sp. infections of cranes
381 (Novilla & Carpenter, 2004). When considering evidence from closely-related *Eimeria*
382 species of cranes, natural infection in all affected organs is frequently sub-clinical, although
383 disease (disseminated visceral coccidiosis) occurs in some cases (Novilla & Carpenter,
384 2004).

385 Future uses of the GeneScan PCR primers as a diagnostic tool could include analysis of
386 faecal samples. In the context of the CRP, this could, first, have established whether the
387 chicks were being exposed to the parasite at all prior to transfer to the pre-release pens;

and, second, have enabled the prevalence of infection in juvenile birds that were ultimately released to be compared to the prevalence in individuals submitted for PME.

Implications for the Corncrake Reintroduction Project

Disease management practices, including prophylactic toltrazuril treatment, appeared to successfully suppress the occurrence of coccidiosis in corncrakes reared for the CRP and maintain infection at a predominantly sub-clinical level. Where drug treatment is considered necessary, treatment of birds with an ionophore-based anticoccidial – which allows a certain proportion of coccidia 'leakage' and development of natural immunity – could be considered preferable to our current toltrazuril-based strategy, however, ionophore drugs typically require milling into food as an additive, a strategy that was not practicable in the CRP.

With increasing evidence for the development of drug resistance in *Eimeria* (Sharman *et al.*, 2010; McDonald & Shirley, 2009), and assuming chicks are immunologically naïve to the parasites whilst in their quarantined early-stage rearing facilities, an alternative control strategy would be to vaccinate chicks with a controlled level of oocysts (Sharman *et al*., 2010) at this early rearing stage. By allowing juvenile corncrakes to encounter *Eimeria* at this younger age, they may develop some immunity to the parasites and therefore be better able to acclimatise to the oocyst burdens encountered in their pre-release aviaries. *Eimeria* sp. infection has been diagnosed at a low level in the CRP's captive breeding stock (Jeanes *et al.*, 2013), and faecal samples from these birds could be utilised in order to expose chicks to the parasite before transfer to the release aviaries. Vaccination is becoming increasingly popular within the poultry industry (McDonald & Shirley, 2009), and indeed is continually progressing in technique (McDonald & Shirley, 2009). However, the persistent infection exhibited by both *E. crecis* and *E. nenei* (Jeanes *et al*., 2013) and by closely related *Eimeria* species (Bertram *et al*., 2015; Yang *et al.*, 2014) in Gruiformes, differ significantly from the hit-and-run characteristics of the *Eimeria* parasites in poultry (Sharman *et al.*, 2010), suggesting that Gruiforme coccidia are likely to be less immunogenic than poultry coccidia.

Exposure of chicks to the parasite would initially need to be highly controlled to identify any pathogenic effects, and to determine the suitable timing and dosage of oocysts. In the short term, vaccination of chicks would be a more expensive and unpredictable option when compared to drug treatment, needing further investigation and ethical approval prior to any trial being undertaken (Shirley *et al*., 2007).

In conclusion, our results are evidence of 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 DNA specific to these species of *Eimeria* in liver +/- spleen provides support for an enzootic extra-intestinal pathogen lifecycle similar to that seen in *Eimeria* species infecting cranes (Novilla & Carpenter, 2004). This finding further supports the hypothesis for *Eimeria* evolution in Gruiformes, whereby its life cycle evolved as a means to compensate for the host species' migratory behaviour (Novilla & Carpenter, 2004).

Acknowledgments

The disease risk management work for the CRP was undertaken by the Disease Risk Analysis and Health Surveillance (DRAHS) team, which is part of a Natural England/ZSL partnership and part-funded by RSPB and the People's Trust for Endangered Species. Project funding was provided by ZSL and the Royal Veterinary College as part of the Masters in Wild Animal Biology course. We are grateful to colleagues from DRAHS, including Fieke Molenaar, Rebecca Vaughan-Higgins, Timothy Hopkins and Jenny Jaffe, as

well as ZSL's Veterinary Services department, ZSL Whipsnade Zoo, PCT, and the RSPB, for their contributions to this research.

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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527 **Table 1.** Summary of tissue samples admitted to this study. Tissue types available for each
528 case are cross-referenced with results of diagnosis following histological examination
529 regarding *Eimeria* infection status.

Histology	Histological diagnosis	No. cases available	Unsuccessful gDNA extraction	Cases admitted to study	Tissue samples available		
					Spleen, liver and intestine	Liver and intestine	Intestine only
Yes	Uninfected	18	1	17	0	0	17
	Coccidia infection only	9	0	9	0	0	9
	Coccidiosis (coccidia-associated enteritis)	14	0	14	2	12	0
No		24	8	16	0	0	16
	Total	65	9	56	2	12	42

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532

533 **Table 2.** Summary of birds found to be infected with each species of Eimeria using 18S
534 rRNA gene primers (ERIB1 and ERIB10) coupled with direct amplicon sequencing, and TTA
535 indel primers (ERIB1 and ERIBn) coupled with GeneScan resolution. The number of
536 samples tested positive is shown in parentheses to include examples where multiple
537 samples from single birds tested positive.

Target DNA region	No. of birds (no. of tissue samples)			
	Eimeria detected	*Eimeria crecis*	*Eimeria nenei*	Co-infected
18S	54 (70)	45 (60)	20 (22)	11 (12)
TTA indel	54 (70)	45 (60)	24 (26)	15 (16)

538

539

Figure legends

Figure 1. Schematic of the positions of primers ERIB1, ERIB10 and ERIBn, and the TTA indel, within the *Eimeria* rRNA sequence repeat (adapted from Honma et al. (2011)).

Figure 2. 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 3. 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 *Eimeria crecis*, as shown by a blue arrow at 180 bp peak. (c) Samples containing *Eimeria 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.

Figure 4. Occurrence of *Eimeria crecis* and *Eimeria nenei* in intestine, liver and spleen samples from juvenile captive reared corncrakes which died whilst in pre-release pens, in association with coccidiosis (confirmed on histopathology). All birds with a positive diagnosis in the liver also tested positive in the intestine. All birds with a positive diagnosis in the spleen also tested positive in the liver and intestine.

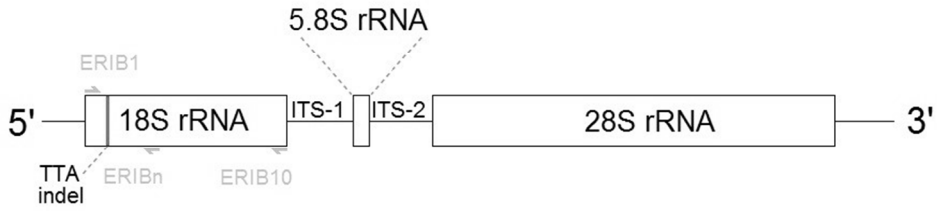


Figure 1. Schematic of the positions of primers ERIB1, ERIB10 and ERIBn, and the TTA indel, within the *Eimeria* rRNA sequence repeat (adapted from Honma et al. (2011)).

247x70mm (96 x 96 DPI)

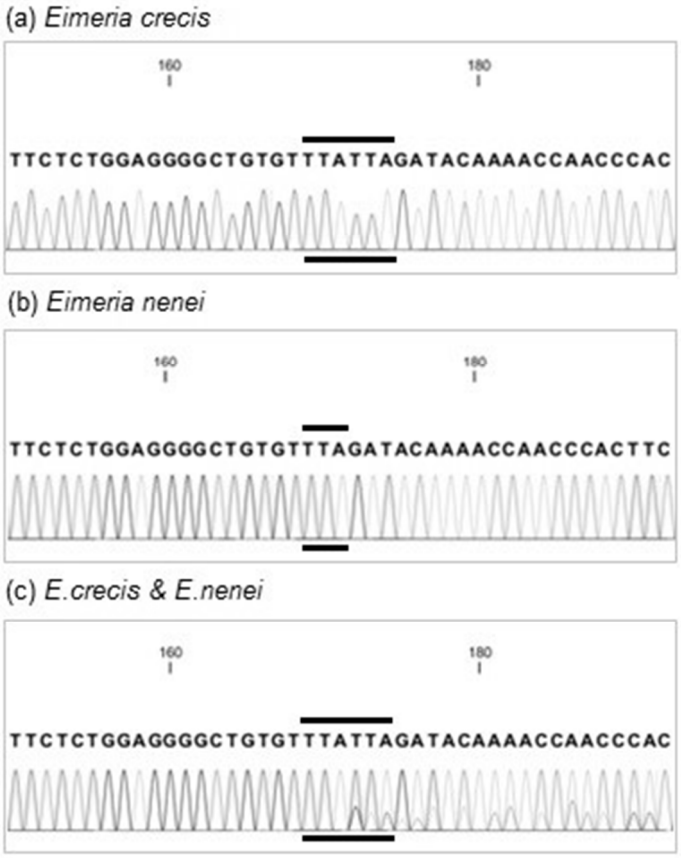
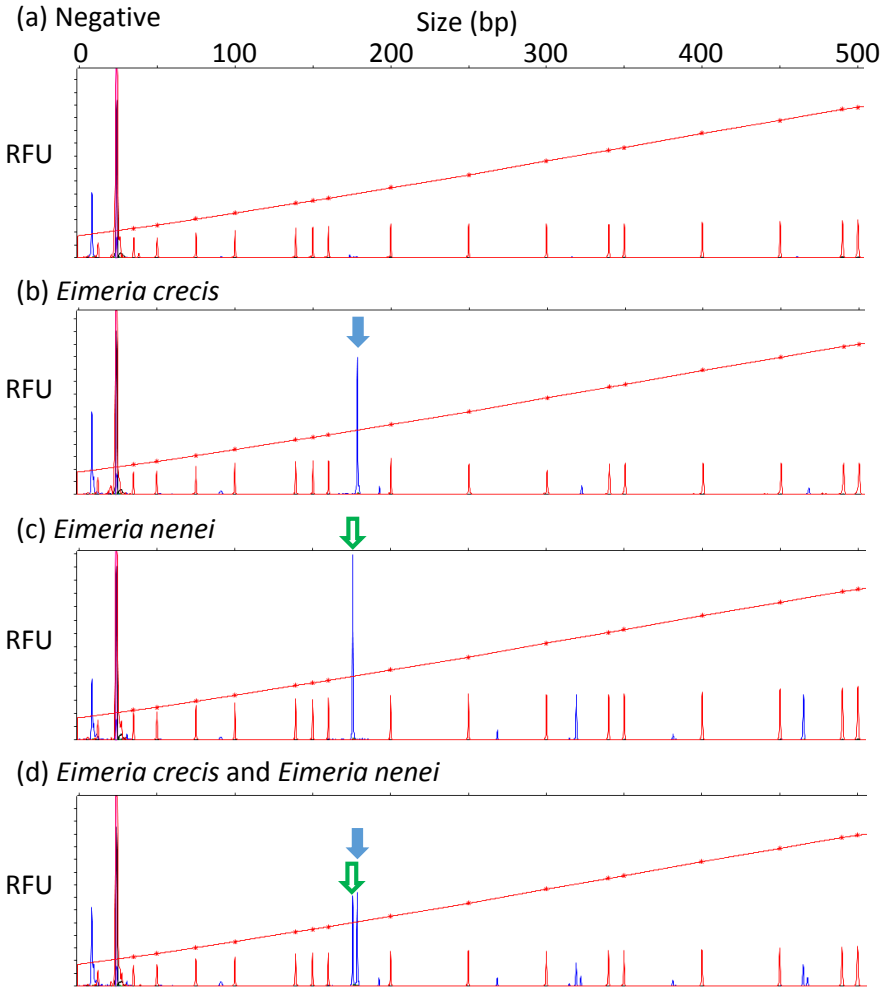
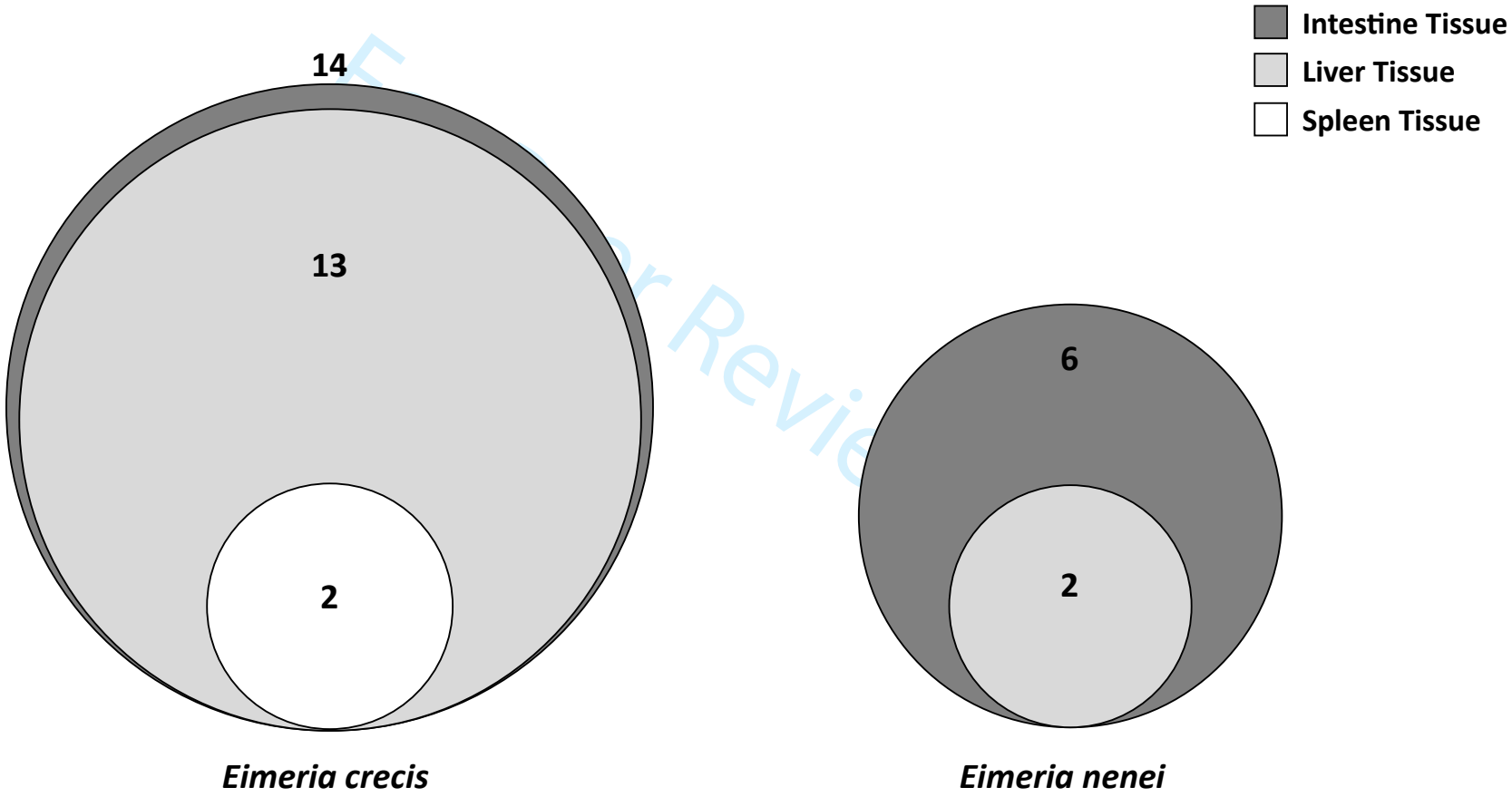


Figure 2. Sequence traces viewed using CLC Main Workbench version 5.7.1 (CLC Bio, Prismet, Denmark). The TTA indel is highlighted by a bold line above and below the trace. (a) The *E. crecis* trace includes the TTA repeat. (b) The *E. nenei* trace has no TTA repeat. (c) Both *E. crecis* & *E. nenei* 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.

94x119mm (96 x 96 DPI)


(a) Negative
Size (bp)
0
100
200
300
400
500
RFU
(b) Eimeria crecis
RFU
(c) Eimeria nenei
RFU
(d) Eimeria crecis and Eimeria nenei
RFU


Intestine Tissue
Liver Tissue
Spleen Tissue
14
13
2
6
2
Eimeria crecis
Eimeria nenei