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Renal trematode infection due to *Paratanaisia bragai* in zoo housed Columbiformes and a red bird-of-paradise (*Paradisaea rubra*)[☆]



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ABSTRACT

Trematode infections affect a diverse range of avian species and the organs that are parasitised are also very varied. The family Eucotylidae contains seven genera of renal flukes that parasitise various birds. In birds, mild to severe lesions have been reported for species of the genus *Paratanaisia*, which was originally described from columbiform and galliform specimens collected in South America and has been identified in a number of wild avian species.

This paper investigates eight cases of renal trematode infection at Chester Zoo in the UK due to *Paratanaisia bragai* in five previously unreported species: red bird-of-paradise, Socorro dove, Mindanao bleeding heart dove, laughing dove and emerald dove. Pathological changes, which varied between species, are discussed. A known intermediate snail host *Allopeas clavulinum* was present in the enclosures but there was no direct evidence of trematode infection. The size of the snails, possible low prevalence and the difficulty of visualising sporocysts contributed to this. Thus the development and application of further molecular diagnostic markers that can be applied to snail tissues is warranted. Parasite identification was confirmed utilizing DNA amplification from formalin-fixed paraffin-embedded tissues using PCR and trematode specific primers. Sequencing full ssrDNA and D1–D3 lsrDNA confirmed the identity in all cases as *P. bragai*. However, the short 310 bp fragment used provides insufficient variation or sequence length for wider application. The epidemiology, pathology and consequences for the management of these endangered species are discussed. Preliminary work on developing an effective ante mortem diagnostic PCR test kit is also highlighted.

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Introduction

Trematode infections affect a diverse range of avian species and the organs that are parasitised are also very varied, including mesenteric veins (Van Bolhuis et al., 2004), the biliary tract (Murata et al., 1998), the intestinal lumen (McJunkin et al., 2003), nasal cavities (Horák et al., 2002), air sacs, oviducts and the corneal surface (Joyner, 1994; Macwhirter, 1994). Parasitism of critical organs such as the kidneys, which have such a limited ability for regeneration, could be a significant cause of morbidity in some species. Avian kidneys can be infected by flukes of several genera of the trematode families Eucotylidae and Rencolidae, with the parasites occurring in the distended renal pelves and proximal ureters

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and adjacent epithelial atrophy, even in individuals with a moderately heavy fluke burden (e.g. see Pinto et al., 2004 and Brener et al., 2006). However, in the family Psittacidae (Luppi et al., 2007) or those which are more geographically isolated, such as Mauritian pink pigeons, *Columba mayeri*, (Bunbury et al., 2008), parasitism by renal flukes may result in a granulomatous interstitial nephritis, and ultimately lead to chronic renal failure and death (Luppi et al., 2007; Bunbury et al., 2008).

This paper discusses eight cases of renal trematode infection, diagnosis and management in four species of captive Columbiformes and a red bird-of-paradise, *Paradisaea rubra* (Passeriformes). All renal trematodosis cases occurred at Chester Zoo in the UK from June 2003 to December 2010. The columbiform species affected by parasitism included: the Socorro dove, *Zenaida graysoni*, a Mexican island endemic species classified as extinct in the wild by IUCN ($n = 3$); the laughing dove, *Stigmatopelia senegalensis*, a widespread species distributed in Africa, the Middle East and southern Asia ($n = 1$); the emerald dove, *Chalcophaps indica*, another widespread species ranging from India to Australia, ($n = 1$); the Mindanao bleeding heart dove, *Gallicolumba crinigera*, a species classed as vulnerable (IUCN, 2011) from the Philippines ($n = 2$). Red birds-of-paradise are endangered endemic Indonesian frugivorous passerines (Worth et al., 1990, <http://www.birdlife.org/datazone/speciesfactsheet.php?id=5840>). The significance of observed disease, and improving ways to diagnose infection ante-mortem, are discussed.

Materials and methods

Pathological investigation

Avian carcasses underwent a complete necropsy on the day of death by following standardised protocols (Keymer, 2000). Tissue samples were obtained from new post-mortem material, as it became available and also from 10% formalin fixed archived tissue samples from historic cases at Chester Zoo. A standard range of tissues (including the kidneys) were sampled for bacterial culture, parasitology and histopathology.

Identification of snails and trematodes from Chester Zoo

Ten live snail (putative intermediate host) samples were collected from bird enclosures, and transported live to the Natural History Museum in London (NHM) for identification. The identity of parasites removed from freshly autopsied renal tissues was confirmed using the NHM reference material of adult eucotylid worms kept on fixed stained and mounted slides, including recently supplied reference specimens of *Paratanaisia bragai* from helmeted guinea fowl collected in Brazil, (slides 2012.4.27.1-6; NHM Parasitic Worms).

Molecular analysis of trematodes and their detection in FFPE samples

Complete 18S and partial (D1–D3) 28S nuclear RNA genes (*ssrDNA* and *lsrDNA* respectively) were PCR amplified and sequenced from a frozen trematode retrieved from renal tissue of a Socorro dove, and an ethanol-fixed specimen of *P. bragai* (ex. Helmeted guinea fowl) from Brazil. Established techniques of DNA extraction, PCR amplification and direct Sanger sequencing were used (e.g. Olson et al., 2003). Sequences were aligned by eye to a previously assembled alignment of *ssrDNA* and *lsrDNA* (Olson et al., 2003) which included *Renicola* sp. and *Tanaisia fedtschenkoi* (Eucotylidae), using MacClade (Maddison and Maddison, 2005). A published sequence of *lsrDNA* from the eucotylid *Tamerlania*

zarudnyi (GenBank accession AF184248) was included in the *lsrDNA* alignment.

To extract DNA from formalin fixed paraffin embedded (FFPE) renal tissues, approx. 2–5 mm³ of sample was removed from the centre of each paraffin block prepared for histology, and DNA extracted using QIAmp DNA FFPE Tissue Kit (Qiagen, UK), following the manufacturer's recommended protocols.

In order to develop PCR primers capable of selectively amplifying short (200–500 bp) fragments of eucotylid or renicolid rDNA, Geneious 5.1 (Primer3 algorithm) was used to predict possible primer pairs and visualise regions of high sequence variability amongst eucotylids and renicolids for both *ssrDNA* and *lsrDNA* data sets across a wide trematode diversity (Olson et al., 2003). Of the primer pairs predicted, sequence variation within *ssrDNA* was deemed too low to be able to differentiate trematode species adequately, and might also amplify bird host rDNA. From a variety of primers designed and tested against *lsrDNA*, a single pair amplifying 310 bp (including primers) was selected and established experimentally as effective for amplifying and sequencing ethanol-fixed eucotylid/renicolid material. *In silico* analyses suggested these primers would not amplify host (vertebrate) rDNA. The same primer pair was used with FFPE DNA samples; primers were forward Para28S_F (5'-AAGCCTGTGCCACTTGGTC-3') and reverse Para28S_R (5'-CGTGCTGTTACCCTCTCTTC-3'). The reverse primer fell in a highly variable region of the trematode *lsrDNA* alignment and was therefore likely only to amplify eucotylids. PCRs were set up using PuReTaq Ready-To-Go PCR Beads (GE Healthcare) under the following conditions: initial denaturation for 5 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 53 °C and 1 min at 72 °C, and completed by 7 min at 72 °C. All successfully amplified genes were directly sequenced, in both directions, with the original amplification primers.

Newly sequenced genes were aligned with appropriate trematode data sets (see above), for comparison with the reference sequences of *P. bragai*, and for quick phylogenetic assessment using neighbour joining (NJ) as implemented in SeaView v.4 (Gouy et al., 2010). Prior to NJ analysis the alignment was run through Gblocks (Castresana, 2000), using default parameters, as implemented in SeaView to remove ambiguously alignable positions.

Results

Case reports

A seven-year-old female red bird-of-paradise, presented in late 2007 with paresis in the right leg, and a poor grip reflex. Haematology and blood biochemistry initially showed minimal changes (ISIS, 2009). Parameters including uric acid and white blood cells increased as the animal's clinical condition deteriorated, and response to therapy was limited. Progressive chronic renal failure was diagnosed with a poor prognosis and the bird was euthanased 26 days after presentation.

On gross post mortem examination the bird was in poor body condition, reflected by depleted pectoral muscles and a lack of coelomic fat deposits. The kidneys were irregularly sized with lobules varying multifocally in colour from red-brown to pale tan. The kidney was locally enlarged around the right sciatic nerve. Bilaterally, both stifle and tarsometatarsal-phalangeal joint cavities were mild to moderately enlarged with gout deposits adherent to the joint capsule.

Histopathologically, in the renal medulla, there were moderately dilated collecting ducts, pelves and ureters, occupied by several clusters of gravid trematodes (Fig. 1A). The surrounding urinary tract transitional epithelium was, multifocally, mildly attenuated (atrophic). The kidneys also had a multifocal mild to

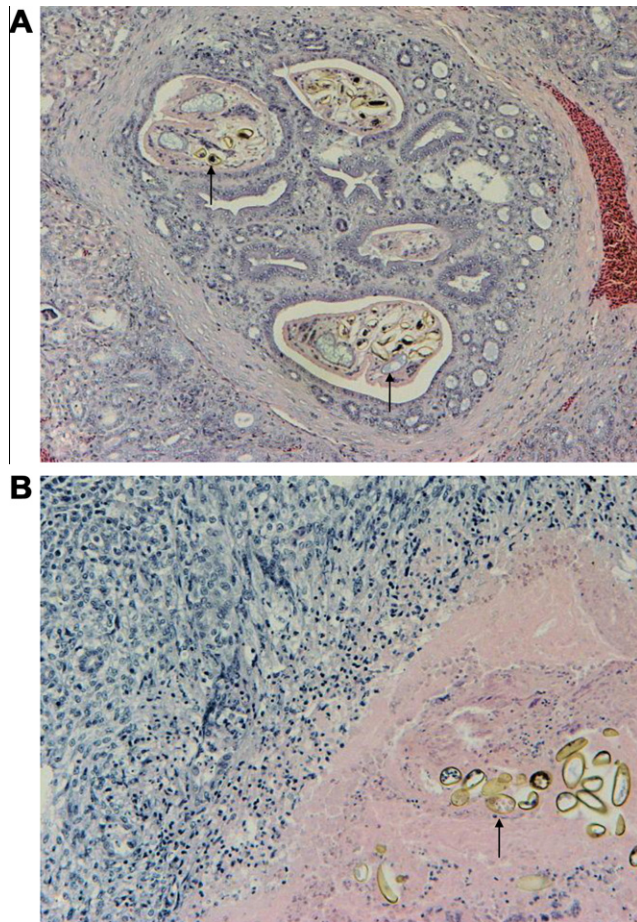


Fig. 1. Red bird-of-paradise case. (A) Renal flukes in collecting ducts (arrows) with minimal inflammatory changes. HE. (B) Necrosis and granulomatous nephritis surrounding trematode eggs (arrow). HE.

moderate granulomatous and fibrosing interstitial nephritis, associated with degenerate adult trematodes and their eggs (Fig. 1B). Moderate numbers of macrophages, haemosiderophages, lymphocytes and multinucleate giant cells were scattered throughout the interstitial tissue but were especially prominent at sites where free eggs were present surrounding the remains of necrotic trematodes. Occasional tubular lumens contained homogenous eosinophilic fluid (proteinaceous) or grey urate material. Multifocally, there were minor areas of interstitial tissue or tubular epithelium dominated by granulomatous inflammation centred around urate crystal deposits.

On microscopic dissection, approximately 40 trematodes were present in the cranial third of the right kidney.

The final diagnosis was made of a mild to moderate multifocal granulomatous interstitial nephritis leading to chronic renal failure associated with the trematode infection. The resulting chronic renal insufficiency precipitated an acute failure with dehydration and articular urate crystal deposition, a common sequel to dehydration in birds (Phalen, 2000).

Fixed, stained and mounted specimens of the trematodes were grossly identified as *P. bragai* (Pinto et al., 2004). Identification was further confirmed by comparison with freshly fixed, stained and mounted specimens of *P. bragai* from Brazil. Subsequent cases from Columbiformes with renal trematodosis induced renal failure included three Socorro doves, one in November 2009 and two in 2010 (June and November). The three doves affected were juvenile birds, considerably younger than the majority of the other columbiform specimens affected, and in poor body condition. The 2009

dove did not have any gross renal pathological lesions however histologically, the kidneys had moderate to severely dilated collecting ducts, pelves and ureters, within which there were numerous clusters of gravid trematodes. The surrounding urinary epithelium was mildly to moderately hyperplastic or atrophic (depending on the degree of dilation) and the surrounding interstitial tissue was infiltrated by mild to moderate numbers of lymphocytes and macrophages.

Both 2010 Socorro doves had much more pronounced gross renal lesions which included moderate irregular enlargement and multifocally mottled pale red to cream parenchyma and dilated ureters. At the cranial renal poles the renal parenchyma was pale cream to dark red and multifocally cystic (Figs. 2A and 3A). A few flecks of white urate crystals were multifocally present in the renal parenchyma around the ureters. There were crystalline gout deposits diffusely lining the pericardial sac (Fig. 2A right) and focally in the lower right hepatic lobe. Histologically, the kidneys had moderate to severe multifocal distension and dilation of the collecting ducts and the ureters by numerous gravid flukes. The collecting duct epithelium was flattened due to pressure atrophy and most ducts were severely dilated and cystic, containing proteinaceous fluid and multifocal basophilic mineral deposits (dystrophic calcification) (Figs. 2B and 3B). Other dilated collecting ducts contained numerous fluke eggs surrounded by cellular debris, numerous macrophages and multinucleate giant cells (Fig. 3C). The renal medulla and cortical interstitial tissue was also multifocally, mild to moderately infiltrated by degenerate and

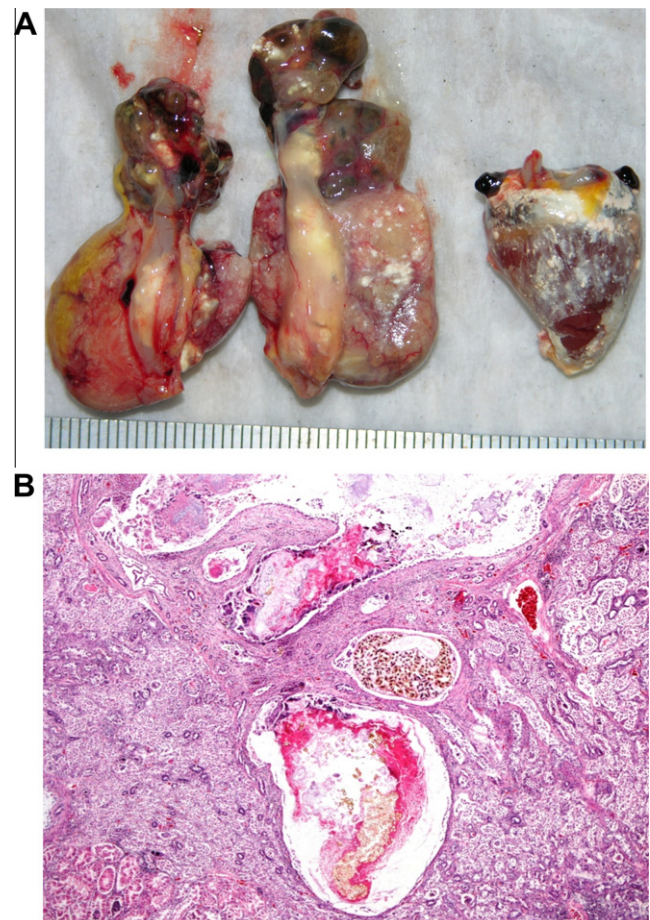


Fig. 2. Socorro dove case 1. (A) Kidneys with cranial atrophy, haemorrhage and caudal polar gout deposition and also pericardial gout deposition. Macroscopic view. (B) Cystic dilation of collecting ducts, haemorrhage and granulomatous nephritis. HE.

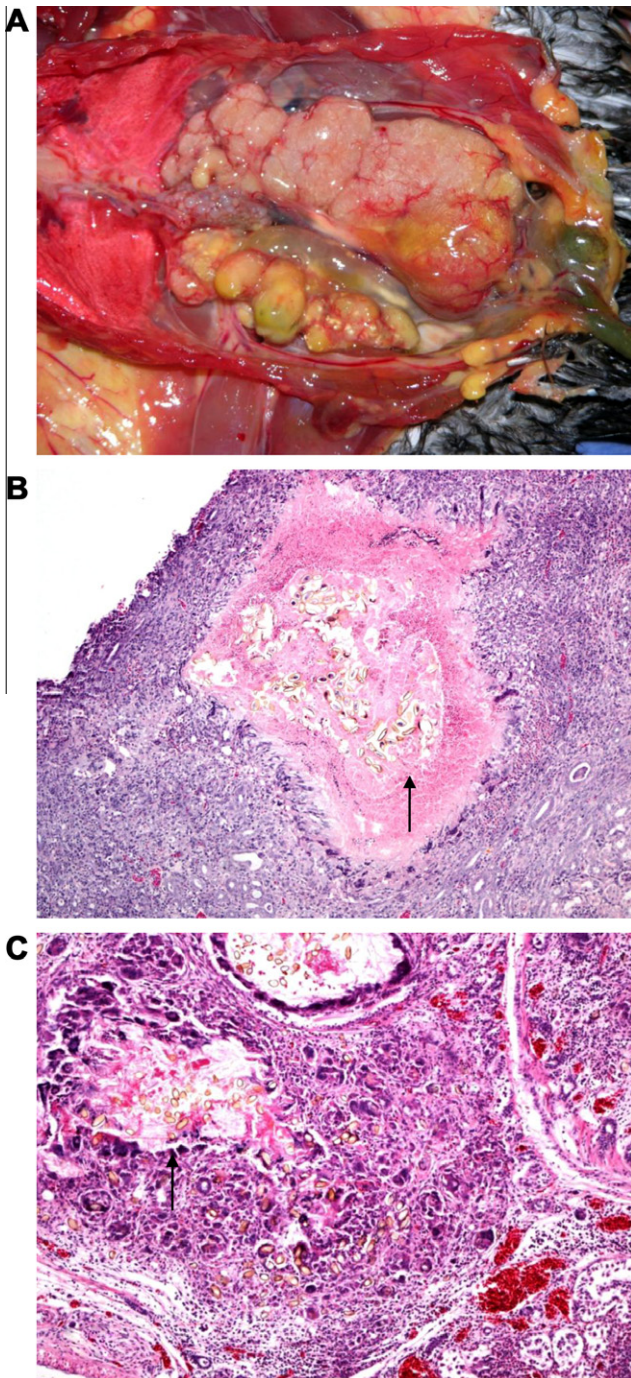


Fig. 3. Socorro dove case 2. (A) Asymmetrically enlarged pale tan left and atrophic / cystic right kidney. Macroscopic view. (B) Kidney with focally extensive central necrosis surrounding fluke eggs (arrow) and surrounding granulomatous inflammatory reaction. HE. (C) Kidney with chronic granulomatous tubulointerstitial nephritis surrounding free trematode eggs. HE.

viable heterophils, lymphocytes and plasma cells. In places, numerous macrophages and multinucleate giant cells were clustered around central areas of necrosis containing free fluke eggs and multifocal tubular lumens were expanded by gout topi (Fig. 3B).

A review of the histological archive from Chester Zoo in addition to ongoing clinical surveillance, revealed a further four cases in three species (Table 1). These were a laughing dove, an emerald dove and two Mindanao bleeding heart doves. All were less severely affected than the bird-of-paradise and Socorro doves, with

minimal or no gross lesions but flukes were identified histopathologically and subsequent PCR (in the Mindanao bleeding heart doves), was used to identify the trematodes.

In these minimally affected cases, the bleeding heart doves both had a dilated collecting duct containing a single gravid trematode with the surrounding epithelium showing mild focal hyperplasia. The underlying interstitial tissue was mildly infiltrated by lymphocytes and plasma cells.

Similarly, the laughing dove and the emerald dove had several fluke parasites distending and expanding the renal pelvis and ureters which were consistent with *Paratanaisia* species. The adjacent ureteric epithelium was mildly hyperplastic and the submucosa contained several macrophages, lymphocytes and plasma cells (Fig. 4), in addition to multifocal mildly degenerate epithelium.

In all cases trematode morphology and molecular structure were consistent with *P. bragai*, indicating that all cases were infected with this species of fluke.

Molecular results

Full *ssrDNA* and partial (D1–D3) *lsrDNA* from the Socorro dove trematode have been deposited with GenBank under accession numbers: JX231100 and JX231099 respectively. Partial *lsrDNA* from the Brazilian isolate of *P. bragai* has been deposited under accession number JX231098. A pairwise alignment of 1257 positions revealed 3% difference between the *lsrDNA* sequences. Neighbour joining analysis each of *lsrDNA* and *ssrDNA* alignments with other trematodes (from Olson et al., 2003) confirmed the close association between the *P. bragai* isolates and their position within the Eucotylidae, grouping with *Tanaisia* and *Tamerlania* species (trees not shown).

PCR amplification of FFPE avian renal tissues targeted to amplify the 310 bp fragment of *lsrDNA* was done for all reported cases except the emerald dove, and yielded positive results for all but one of the samples. The failure of a successful PCR amplification in the FFPE sample from the laughing dove may be that insufficient or no parasite DNA was removed when the small sample was taken from the histology block. For the five cases where amplicons were generated, sequencing confirmed the identity of all flukes as the same species extracted and sequenced from the Socorro dove, confirmed morphologically as *P. bragai*. The full (D1–D3) sequence of *lsrDNA* from Brazil (ex helmeted guinea fowl) differed from the Socorro dove specimen by 3%; 1257 bp. Each of the 319 bp fragments sequenced from positive FFPE samples sequenced identically to one another and to the samples from the Socorro dove, all from Chester Zoo. The Chester Zoo isolates were 2 bp different from the *P. bragai* specimen from Brazil. This same region differs between *P. bragai* and *T. fedtschenkoi* (AY116870) by 2 bp, and *T. zarudnyi* by 4 bp; see Table 3. These small differences provide evidence of eucotylid infection and, in the light of sequencing full *ssrDNA* and D1–D3 *lsrDNA*, confirm the identity between Chester Zoo samples (= *P. bragai*). However, the short 310 bp fragment provides insufficient variation or sequence length for wider application.

Collection of snails to identify putative intermediate hosts

The conical shelled *Allopeas clavulinum*, (Subulinidae) (Fig. 5), a known intermediate host of *P. bragai* and a further subulinid, *Subulina octona* were morphologically identified from enclosure searches. Two other potential snail intermediate hosts were tentatively identified as *Oxychilus draparnaudi* and *Zonitoides arboreus*. Further molecular characterization and dissection of fresh, or freshly preserved specimens is required for more accurate identification. *S. octona* and *A. clavulinum* are tropical snails with global distribution in greenhouses. *O. draparnaudi* is endemic to Europe and *Z. arboreus* is a greenhouse pest with a global temperate

Table 1
Summary of cases, including predominant renal lesions and role in mortality; m - month.

Signalment	Date of Death	Cause of Death	Max number of flukes/ $\times 40$ field	Main renal histopathology	Secondary histopathological lesions
Mindanao bleeding heart dove, 5 year female. Aviary 1.	30.7.03	Egg yolk coelomitis	1	Focal mild interstitial nephritis around ducts with flukes	Isolated focal necrosis
Laughing dove, >8 year female. Aviary 1.	28.2.06	Trauma	6–7	Marked dilation of ducts	No abnormality detected
Red bird-of-paradise, 7 year female. Aviary 2.	28.11.07	Renal failure	4	Multifocal moderate interstitial nephritis around ducts with flukes	Multifocal moderate necrogranulomas around free eggs
Socorro dove, 4 m male. Aviary 2.	11.11.09	Renal failure	15–20	Multifocal moderate interstitial nephritis around ducts with flukes. Marked dilation of ducts	No abnormality detected
Mindanao bleeding heart dove, 8 year male. Aviary 1.	1.2.10	Undetermined	1	Multifocal mild to moderate interstitial nephritis around ducts with flukes	No abnormality detected
Socorro dove, 11 m male. Aviary 1.	14.6.10	Renal failure	10	Multifocal moderate interstitial nephritis around ducts with flukes. Marked dilation of ducts	Multifocal moderate necrosis around gouty tophi
Socorro dove, 13 m female. Aviary 1.	26.11.10	Renal failure	None found, just eggs	Diffuse chronic severe pyogranulomatous tubulointerstitial nephritis	Multifocal moderate necrosis around gouty tophi
Emerald dove, 7 m female. Aviary 1.	16.12.10	Self inflicted trauma – window strike/collision.	15–20	Dilated collecting ducts with mild epithelial hyperplasia. Interstitial tissue infiltrated by lymphocytes	No abnormality detected

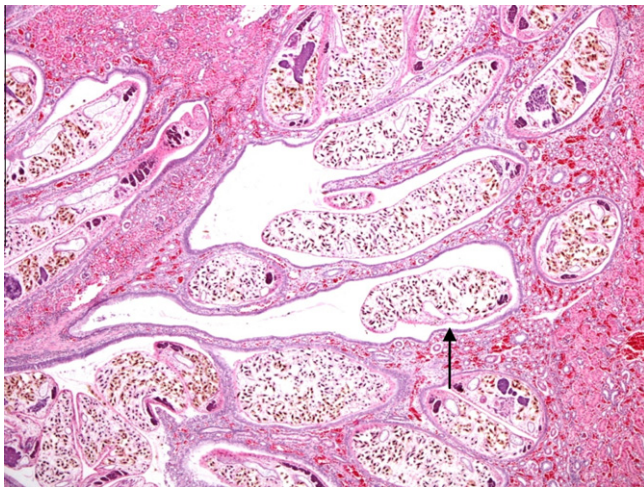


Fig. 4. Emerald dove case: Kidney with dilated collecting ducts and numerous cross section of trematodes (arrow) with minimal inflammation. HE.

distribution. Insufficient numbers and poor preservation of these other snails prevented checking for parasitoses.

Discussion

Table 1 provides an overview of all case reports. This is the first reported case of renal trematodosis in a red bird-of-paradise. However, the role of the trematodes in inducing the severe, chronic renal failure and urate crystal deposition remains open. Histologically there were areas where there was an inflammatory/fibrous tissue response to trematode infection but this was not universally applicable and in some areas the infection appeared incidental. Inflammation was most marked around free trematode eggs yet was generally minimal around the adults suggesting a degree of local immunotolerance. The lameness seen in this bird could have been associated with pressure on or constriction of the lumbar and sacral nerve plexi, which are closely associated with the kidneys. Nerve plexi damage often occurs in renal

disease in birds and this can result in unilateral non-weight bearing lameness (Echols, 2006).

Endoparasite infections have been investigated in birds-of-paradise, both in ex-situ captivity (Zerbe et al., 2009) and *in situ* captivity (Varghese, 1987), but renal trematodes have not been previously reported.

It is likely that this bird-of-paradise gained the infection while in its current enclosure. Eucotylid larval development is completed 40 days post infection, with encystment of the metacercaria (Brandolina and Amato, 2006). In the bird-of-paradise samples, the infection appears well established with predominantly young adult trematodes, indicating the infection is most likely to have been contracted within the previous few months. All three immature Socorro doves exhibited pronounced histopathological renal lesions which were directly attributable to the *P. bragai* infection. All cases had cystic dilation of the renal tubules/collecting ducts and a severe granulomatous nephritis especially around free eggs in the interstitial tissue. Unlike the bird-of-paradise, this trematode infection appeared to be a significant cause of mortality in the young Socorro doves producing such severe histopathological renal lesions that kidney failure was directly attributable to the flukes. This granulomatous nephritis was more pronounced than that in the bird-of-paradise and present in younger birds, suggesting this species is highly susceptible to infection with this parasite. Island endemic species are typically susceptible to disease induced by novel infections as demonstrated by another critically endangered island pigeon species, the Mauritius pink pigeon which has also been identified with this parasite resulting in similar lesions (Bunbury et al., 2008). However, not all Socorro doves examined histologically within the time period of the study had renal lesions and another three doves tested from renal tissue by PCR were negative for infection (see appendix). These other three Socorro doves were all adults and were examined by post mortem and histopathology at least 2 years earlier than the first *Paratanaisia* case in a juvenile Socorro dove. In addition, three pink pigeons were negative for flukes histologically. This could suggest that the fluke infection may be less severe in adult doves, possibly as they are less likely to eat snails or alternatively, the infection may not have been present in the Socorro dove enclosure at that time, although it was in the collection overall as demonstrated by the first Mindanao

Table 2Recorded infections of *Paratanaisia bragai* in wild and *captive birds. IUCN status given (see bottom of table for abbreviations).

Species	IUCN	FFPE	Infection	Native range of bird; [zoo location]	Reference
<i>Columbiformes</i>					
*Mindanao bleeding heart dove <i>Gallicolumba crinigera</i>	VU	+ve	Subclinical	Philippines; [Chester, UK]	This study
*Laughing dove <i>Stigmatopelia senegalensis</i>	LC	–ve	Subclinical	Sub-Saharan Africa, Middle East, SE Asia to India; Western Australia; [Chester, UK]	This study
*Socorro dove <i>Zenaida graysoni</i>	EW	+ve	Fatal	Island of Socorro (Pacific) but extinct in wild; [Chester, UK]	This study
Ruddy ground dove <i>Columbina talpacoti</i>	LC		Not fatal	New World tropics (S. America, Trinidad & Tobago)	Pinto et al. (2004)
*Jambu fruit dove <i>Ptilinopus jambu</i>	NT	+ve	Subclinical	Thailand, Malaysian Indonesia; [Chester, UK]	This study
*Red throated ground dove <i>Gallicolumba rufigula</i>	LC	+ve	Subclinical	West Papua, Indonesia, Papua New Guinea; [Chester, UK]	This study
*Luzon bleeding heart dove <i>Gallicolumba luzonica</i>	NT	+ve	Fatal	Luzon and Polillo Islands, Philippines; [Chester, UK]	This study
Domestic pigeon <i>Columba livia f. domestica</i>	DOM		Not fatal	Global – infection in Brazil Venezuela, Philippines, Puerto Rico, India	Many reports, for example: Maldonado and Hoffman (1941), Tubangui and Masiluñgen (1941), Nasir and Diaz (1972), Borah et al. (2009) Bunbury et al. (2008)
Mauritian pink pigeon <i>Columba mayeri</i>	EN		Fatal	Mauritius	
<i>Galliformes</i>					
Chicken <i>Gallus gallus domesticus</i>	DOM				
Spot-winged wood quail <i>Odontophorus capueira</i>	LC		Not fatal	Global – infection in Brazil South America	Santos (1934) Muniz-Pereira et al. (2009)
Common pheasant <i>Phasianus colchicus</i>	LC		Not fatal	Native to Caucasus and Russia, introduced worldwide; cases – Brazil, Cuba	Gomes et al. (2005)
Turkey (wild and domestic) <i>Meleagris gallipavo</i>	LC/DOM		Not fatal	Native range: N. America. Domestic case – Brazil	Barretto and Mies Filho (1934), cited in Stunkard (1945), Brener et al. (2006) Menezes et al. (2001)
Helmeted guinea fowl <i>Numida meleagris</i>	LC		Not fatal	Sub-Saharan Africa; introduced West Indies, Brazil and France. Case: Brazil	
<i>Passeriformes</i>					
*Red bird-of-paradise <i>Paradisaea rubra</i>	NT	+ve	Contributory to renal disease	Indonesia; [Chester, UK]	This study
Ovenbird <i>Seiurus aurocapillus</i>	LC		Not fatal	Infection in N. America but winters in Caribbean and S. America	Byrd and Denton (1950)
Swainson's warbler <i>Limnothlypis swainsoni</i>	LC		Not fatal	USA, winters in West Indies and Yucatan	Byrd and Denton (1950)
Bronzed grackle <i>Quiscalus quiscula aeneus</i>	LC		Not fatal	N. America	Byrd and Denton (1950)
Double toothed barbet <i>Lybius bidentatus</i>	LC		Fatal	N. America	Rotstein et al. (2003).
<i>Anseriformes</i>					
Black-bellied whistling duck <i>Dendrocygna autumnalis</i>	LC		Not fatal	Southernmost USA, Central & S. America	Fedynich et al. (1996)
Fulvous whistling duck <i>Dendrocygna bicolor</i>	LC		Not fatal	Central & S. America, Sub-Saharan Africa and India	Fedynich et al. (1996)
<i>Psittaciformes</i>					
*Blue-winged macaw <i>Primolius maracana</i>	NT		Fatal	South America; [Belo-Horizonte, Brazil]	Luppi et al. (2007)
*White-eared parakeet <i>Pyrhura leucotis</i>	NT		Fatal	Brazil; [Belo-Horizonte, Brazil]	Luppi et al. (2007)

FFPE – pathology samples tested with preliminary molecular (*IsrDNA*) marker (this study): –ve or +ve. Details of fate of birds found infected marked as 'fatal' from pathology cases and (presumed) 'not fatal' from infections found in helminthological surveys. Note: other *Paratanaisia* species have been reported for additional species, including Piciformes and Tinamiformes. IUCN status: EX – extinct; EW – extinct in the wild; EN – endangered; VU – vulnerable; NT – near threatened; LC – least concern; DOM – domesticated.

bleeding heart dove case in 2003. Possibly, this fluke infection has been slowly progressing through enclosures transported by reservoir host doves of other species.

PCR screening of formalin-fixed paraffin embedded tissue blocks from 22 additional birds representing 11 columbiform species including several known possible host species at Chester Zoo was conducted (Supplementary material). From these, 6 cases of *P. bragai* were confirmed; from 1 Luzon bleeding-heart dove, 2 Socorro doves, 1 Mindanao bleeding-heart dove, 1 Jambu fruit dove

and 1 Red throated ground dove. As renal tissue was taken from paraffin embedded formalin fixed blocks and not frozen tissue, there may have been several false negative results obtained, as the formalin fixation will disrupt long chains of DNA and reduce sensitivity. Nonetheless, these results suggest value in screening paraffin blocks, improving molecular diagnostic markers and developing molecular ecological markers.

Other dove species appear to act as carriers. For example the Mindanao bleeding heart doves were minimally affected but could

Table 3
Alignment of 310 bp fragment of 28S rDNA from eucotylid trematodes. Primers (shown lower case) are specific to eucotylids. Samples are Chester Zoo pathology samples of *Paratanaisia bragai*, Brazilian isolate of *P. bragai* and published sequences of *Tanaisia zarudnyi* and *Tamerlania fedtschenkoi*.

P_bragai_Chester	1	aagcctgtgtccacttgggtcACTAGGCAATGTGGTGTTCAGGTCGTTCCGCAGAGACT	60
P_bragai_Brazil	1	-----	60
AY116870_Tanaisia	1	-----t-----T-----	60
AF184248_Tamerlania	1	-----G-----	60
P_bragai_Chester	61	GCTCCACCCTAAGTCCAGCAATGAGTACGGTGGTATGGACATGGCCCATAGAGGGTGAAA	120
P_bragai_Brazil	61	-----	120
AY116870_Tanaisia	61	-----	120
AF184248_Tamerlania	61	-----	120
P_bragai_Chester	121	GGCCCGTAGGGGTGGAGGTTCCGGCATGTCAGTGTCTCTCTGGGTAGACCTTGGAGTCGGG	180
P_bragai_Brazil	121	-----A-----	180
AY116870_Tanaisia	121	-----	180
AF184248_Tamerlania	121	-----T-----C-----	180
P_bragai_Chester	181	TTGTTTGTGAATGCAGCCCAAAGTGGGTAAACTCCATCCAAGGCTAAATACTTGAC	240
P_bragai_Brazil	181	-----T---	240
AY116870_Tanaisia	181	-----G-----	240
AF184248_Tamerlania	181	-----	240
P_bragai_Chester	241	GAGTCCGATAGCGAACAAAGTACCGTGAGGGAAAGTTGAAAAGTACTTTGgaagagagggtaaacagcacg310	
P_bragai_Brazil	241	-----	310
AY116870_Tanaisia	241	-----	310
AF184248_Tamerlania	241	-----	310



Fig. 5. *Allopeas clavulinum* one of two subulinid snails possibly acting as intermediate host for the *Paratanaisia bragai* infection, found in one of the aviaries housing several of the cases.

carry low levels of renal flukes. In contrast the emerald dove examined had a significant trematode infection and limited gross or histological changes suggesting this species would be an ideal reservoir host for the infection.

Ante-mortem diagnosis of renal trematodosis is difficult, as obtaining a urine sample from birds is problematic (Echols, 2006). The bird-of-paradise had been in the collection for 7 months. During this time she had had three parasite checks using

the Modified McMasters technique (Cheesborough, 2005) with no parasites seen. This technique was developed for strongyle infections in ruminants, and, along with salt and sugar flotation methods are considered unsuitable for diagnosing trematode infections (Cheesborough, 2005). Although a sedimentation process such as the formol-ether concentration technique would be useful for gastrointestinal trematodes, this would remain insensitive for renal trematode diagnosis as avian urine is harder to collect, reducing the test sensitivity. Because of this, molecular diagnostic markers would appear to be the most promising, particularly if they can be applied to various substrates including pathology samples, urates, snails and soil.

Morphological examination of stained whole-mounted material and comparison with reference material supplied from guinea fowl in Brazil, strongly supports the identification of the trematode as *P. bragai* in all cases. Molecular results conducted on all cases except the emerald dove are also consistent with this identification, with the D1–D3 *lsrDNA* sequences of the reference *P. bragai* sharing 97% similarity with that from the Socorro dove; the 3% difference is consistent with within-species variation for other taxa (Unpublished observations, DTJL) but wider sampling of *P. bragai* populations is needed to verify this. Analysis of the short *lsrDNA* fragment obtained from the FFPE samples also suggests those PCR positive were also infected by *P. bragai*. However, the family Eucotylidae is in need of systematic revision and would benefit from additional

molecular systematic data from each of its constituent species in order to better resolve and delineate individual eucotylid species, and to better circumscribe *P. bragai*. Given the widespread geographic distribution of reports of *P. bragai* in a taxonomically diverse array of bird hosts, there is also a need for molecular ecological and population genetic studies to determine the origins, movements and propensity for host-switching within the species. Such markers, perhaps from nuclear ITS rDNA or mitochondrial genes (e.g. see Nolan and Cribb, 2005; Gordon et al., 2011) might also shed light on the epidemiology of the parasite within and between infected aviaries.

P. bragai are parasites of the kidney and ureter and affect several bird species, particularly in Latin America (Luppi et al., 2007); see Table 2. It is found in rock pigeons, *Columbia livia*, in renal tubules (Brandolina and Amato, 2007). The large number of trematodes in the current cases is a common pattern of this infection (Menezes et al., 2001; Pinto et al., 2004; Gomes et al., 2005; Brener et al., 2006). However, the severity and pattern of the microscopic lesions seem not to be associated with the size of the worm burden but could be related to the size of the parasites, site of the infection, parasite strain and host. Also, mild lesions can be the result of mechanical action of the parasites (Gomes et al., 2005). The findings of the current study support this.

In species where infection is thought to be aberrant, the course of infection and pathology appears very similar to that seen in the red bird-of-paradise and Socorro dove cases. In blue winged macaws, *Primolius maracana*, and white eared pheasants, *Crossoptilon crossoptilon*, infections were associated with an intense granulomatous nephritis. These birds died due to the renal fluke infection with associated major increases in uric acid, plus a discrete anaemia and leucopaenia (Luppi et al., 2007). In the red bird-of-paradise case, the changes observed in the blood biochemistry and the renal pathological changes due to the parasite support the hypothesis that this is a chronic infection in an aberrant host.

It is highly relevant that this infection is caused by the same trematode species throughout the collection. All cases found were in two aviaries – both extensively planted and naturalistic with various earth and leaf litter substrates and with multiple mixed species, making it an ideal environment for potential trematode intermediate hosts and interspecies infection. Although several species of pulmonate gastropod were collected from these aviaries at Chester Zoo, the two subulinids, *S. octona* and *A. clavulinum* are the most likely vectors for transmitting *P. bragai* to captive birds. In Brazil *S. octona* has been found to be a host for *P. bragai* in the wild, and another subulinid *Leptinaria unilamellata* has been shown to be capable of experimental infection (Maldonado, 1945; Keller et al., 1992). *A. clavulinum* and *S. octona* were some of the most commonly found snail species in the Chester Zoo aviaries. It is possible that infected snails were introduced into the aviaries during replanting as both species are commonly and globally distributed in tropical greenhouses. A cursory investigation of 40 individual snails had no direct evidence of trematode infection and development and application of further molecular diagnostic markers that can be applied to snail tissues, is warranted. Additionally, such markers might be applicable to bird urates and soil samples for detecting and monitoring of infections.

As the snail hosts identified are all small, we cannot discount accidental ingestion by birds not usually expected to feed on snails. However, the female red bird-of-paradise may have been specifically looking for snails to eat. This species is known to have a smaller intake of invertebrates in its day-to-day diet, compared with other bird-of-paradise species (Zerbe et al., 2009). Invertebrate ingestion is anecdotally a common finding around nesting time in this species (Worth et al., 1990), and contributes to increased mineral intake, particularly calcium. No snails were found in the gastrointestinal tract on post mortem. As a predominantly ground

dwelling species, the Socorro dove may also target invertebrates to feed nestlings so increasing both infection likelihood and infectious dose, via ingestion of an intermediate host.

Records of *Paratanaisia* species in zoo housed and wild birds are compiled in Table 2. The global distribution of *P. bragai* confounds our understanding of its origins, its natural and full host range, until we have better sampling and a phylogeny to hand. Given its low host-specificity, its prevalence amongst domesticated birds, and presence in migratory species, a fuller understanding of its host associations and origins is needed. Accurate diagnosis, additional sampling and screening of potential intermediate hosts in both the wild and captive enclosures are required to evaluate further the threat of *P. bragai*. Moreover, given that nine FFPE renal samples from columbiform birds, and one from the red bird-of-paradise yielded positive PCR results for the parasite, it may in future be possible to develop more variable markers for epidemiological purposes, determining the origins of the infection.

This case series shows there is now an endemic mild to severe *P. bragai* infection in the Chester Zoo collection, preferentially affecting Columbiformes and occasionally other species. The cases from bird species detected positively by histopathology probably represented the majority of the infections in this group of species. Nevertheless, increased screening of avian kidneys from at-risk species, predominantly Columbiformes, Galliformes, psittacines and passerines, is warranted. This will monitor the impact of this infection on these rare species more effectively and, in addition, we plan to develop a PCR-based diagnostic for urates which will enable effective ante mortem screening. Both these procedures will aid in the development of more targeted and effective preventative medicine protocols for the species concerned.

Prophylactic treatment was initiated for the remaining red birds-of-paradise, Socorro doves and Mindanao bleeding heart doves in the collection, by administering Praziquantel. A 50 mg tablet was crushed in 2.5 ml of water to provide approximately 20 mg/ml. This was administered at a dose rate of 20 mg/kg either orally to manually restrained birds or the crushed tablet was sprinkled and mixed in feed at 2.5 mg/20 g of feed. Dosing occurs every 3 months. Praziquantel has a narrow spectrum of activity against trematodes, and was deemed the most effective treatment at removing a heavy burden without endangering the bird. There have been no adverse reactions observed with this dosage in other bird-of-paradise species (Hammer, pers comm; Zerbe et al., 2009) or Columbiformes (author's clinical experience). The birds remain clinically healthy, but the actual efficacy of this medication remains undetermined.

Phasmarhabditis hermaphrodita, a microscopic nematode that enters slug and snail bodies and infects them with bacteria that causes fatal disease (e.g. Rae et al., 2009), was investigated for possible biological control of the subulinid snails. This was found to be ineffectual in the intermediate snail hosts found. Future breeding pairs of now known susceptible species will be housed in a snail-free indoor aviary. Mindanao bleeding heart doves will be closely monitored in future to see if there is any evidence for its reservoir status. Emerald and laughing doves have been moved out of the collection and so cannot currently be studied in the collection concerned.

Although this study reports on cases of trematodosis in captive and endangered species there are clearly implications beyond aviary management and veterinary care. Conservation programmes where birds, such as the Socorro dove are being planned for release back into native habitats e.g. Yanga et al. (2011), need to be aware of the risks of accidentally introducing avian renal trematodes to naïve avian populations, particularly if suitable snail hosts are also available in the wild. As noted by Yanga et al. (2011), Socorro doves selected for reintroduction should be screened carefully to evaluate potential immunological challenges to avoid introduction of

diseases that are apparently absent from native Columbiformes on Socorro Island. Their paper describes the extensive ante mortem disease surveillance that they performed on wild columbiform birds on the island, but the techniques used were unlikely to have detected renal trematodes. In this paper, we illustrate the first steps in developing ante mortem diagnostic technology for avian renal trematodosis. We have highlighted and suggested risk mitigation strategies of a captivity acquired parasite with pathological consequences for several endangered species. This will be of direct benefit to the conservation of Socorro doves and other endangered avian species in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijppaw.2012.11.001>.

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