International Journal for Parasitology: Parasites and Wildlife 17 (2022) 211–217

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Fatal toxoplasmosis in Little Penguins (Eudyptula minor) from Penguin Island, Western Australia

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ARTICLE INFO

Keywords:
Little penguin
Toxoplasma
Hepatitis
Splenitis
Haemoproteus
Apicomplexa

ABSTRACT

Routine post mortems of deceased penguins from Penguin Island, Western Australia, found that a temporal cluster of cases presented with characteristic gross and microscopic changes, namely birds in good body condition with hepatomegaly and splenomegaly, multifocal hepatic and splenic necrosis and numerous, 1–2 μm diameter protozoan parasites within the necrotic foci.

Electron microscopy identified the protozoa as belonging to the phylum Apicomplexa. Molecular investigations by PCR gave inconsistent results. PCR performed by an external laboratory identified a novel *Haemoproteus* spp. organism in samples from 4 of 10 cases from this group, while PCR at Murdoch University identified *Toxoplasma gondii* in 12 of 13 cases (including 9 of the 10 assayed at the external laboratory). Immunohistochemistry of formalin fixed tissues also identified *Toxoplasma* in the hepatic and splenic lesions.

The distinctive mortalities which were observed in this group of penguins are attributed to a fulminant toxoplasmosis, with a concurrent *Haemoproteus* infection in some cases. Though the clinical signs of infection are unknown, the gross and microscopic appearance at post mortem is sufficiently characteristic to allow a diagnosis to be made on these features. Definitive confirmation of *Toxoplasma* infection can be made by immunohistochemistry or PCR.

1. Introduction

Little Penguins (*Eudyptula minor*) are the smallest of all penguin species, and the only penguin native to Australia. While the worldwide number of Little Penguins remains plentiful, some regional populations of Little Penguins from a number of locations around Australia have been reported to be in decline (Dann et al., 1996, NSW National Parks and Service, 2003; Bool et al., 2007, Stevenson and Woehler, 2007). In many cases, the causes for these population declines have been attributed to human activities such as habitat alteration, reduction in fish stocks, pollution or entanglement in fishing paraphernalia (Stevenson and Woehler, 2007). Also, onshore nesting sites are vulnerable to predation from introduced species. In Western Australia, they nest mainly on offshore islands, and the largest colony of Little Penguins in Western Australia breeds on Penguin Island, located roughly 40 km south of the city of Perth (Wienecke et al., 1995).

Murdoch University Veterinary Hospital's (MUVH) pathology department has provided diagnostic post mortem services for deceased Little Penguins from Penguin Island since at least 2001. In 2011/2012, 13 penguins were found to have evidence of a protozoan parasite infection, principally affecting the liver and spleen, which caused significant necrosis of these tissues and which was deemed to have caused the deaths of the birds (see Cannell et al., 2013 for the results from 8 of these birds). Retrospective analysis of earlier submissions dating back to 2006 identified only two additional (but isolated) cases with similar gross and histopathological findings (Cannell et al., 2013) (note that archived tissues for these two historical cases were not available, so they were not investigated further in this study). On the basis of the parasite morphology and the pattern of infection predominantly affecting the reticuloendothelial system, each of these cases was provisionally

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https://doi.org/10.1016/j.ijppaw.2022.02.006
Received 4 November 2021; Received in revised form 8 February 2022; Accepted 8 February 2022
Available online 11 February 2022

diagnosed as the haemoparasitic disease avian malaria. Avian malaria is caused by various *Plasmodium* species, has a worldwide distribution (Atkinson et al., 2008) and is common in captive penguin populations.

Avian protozoal parasites include protozoa from the order Haemosporida (e.g., *Plasmodium*, *Haemoproteus* and *Leucocytozoon*) and Piroplasmida (e.g., *Babesia*). Infection of free-living birds with parasites from the Haemosporida is relatively common worldwide, except in Antarctica (Valkiūnas 2004, Ishtiaq et al., 2007, Sturrock and Tompkins, 2008; Quillfeldt et al., 2011, Imura et al., 2012). Many *Plasmodium* species parasites have a low host specificity, and cross-infection between avian species occurs quite commonly (Atkinson et al., 2008). *Haemoproteus* and *Leucocytozoon* parasites, by contrast, tend to be highly host specific (Valkiūnas 2004; Atkinson et al., 2008). For the most part, this means that the avian hosts are relatively well adapted to these parasites and infections are usually subclinical (Peirce et al., 2004; Atkinson et al., 2008). Aberrant infections, however, do occur, and disease and deaths due to haemoproteosis and leucocytozoonosis have been reported in captive birds, especially in mixed aviaries and zoos where birds may be exposed to haemoparasite species which they would not otherwise encounter (Ferrell et al., 2007; Donovan et al., 2008; Olias et al., 2011, Palinauskas et al. 2013). The prevalence of haemoparasite infections in seabirds is somewhat lower than that in land-based bird populations, which has been attributed to reduced exposure to the invertebrate vectors, and to natural immunity (Deem et al., 2010; Ploeg et al., 2011; Quillfeldt et al., 2011). Even so, penguins are among the seabirds which have been found often to have haemoparasite infections (Quillfeldt et al., 2011; Sijbranda et al., 2017). For a comprehensive review of blood parasites affecting penguins, including haemosporidian, piroplasmid and kinetoplastid protozoa, spirochete bacteria, and nematode microfilariae, see Vanstreels et al., (2016). Note that testing of Penguin Island penguin samples conducted at the University of New Mexico (UNM) (results published in Cannell et al., 2013) found no evidence of *Plasmodium* infection. However, 4 of the 10 cases were positive for a novel lineage of *Haemoproteus* in the subgenus *Parahaemoproteus* (GenBank accession numbers KC121053–KC121056).

Among apicomplexan parasites other than the Haemosporida, few have been reported to cause infections in penguins. Probably the most notable is a piroplasmid infection in African Penguins (*Spheniscus demersus*), in which *Babesia peircei* is considered to be enzootic (Earlé et al., 1993). A newly described lineage of *Babesia* has also been detected in Little Penguin populations in the south east of Australia at an estimated prevalence between 3.4% and 4.5%, though this parasite was not associated with disease (Vanstreels et al., 2015).

Another apicomplexan which has been reported to cause disease in a single Little Penguin is *Toxoplasma gondii* (Mason et al., 1991); in this case, the infection resulted in marked hepatitis and splenitis, and was acutely fatal. While it is not a haemoparasite as such, *T. gondii* can invade the nucleated erythrocytes of avians (Tanabe et al., 1980; Malkwitz et al., 2017), a feature which does not occur in mammalian species. Toxoplasmosis has also been reported to cause fatal infections in juvenile African penguins (Ploeg et al., 2011). Though the exposure of seabirds to *T. gondii* would be expected to be low (since the definitive host of this parasite is the cat), a survey of Galapagos Penguins (*Spheniscus mendiculus*) curiously found that those living on an island inhabited by cats (Isabela Island) had a lower rate of seroconversion to *T. gondii* than those living on an adjacent, cat-free island (Fernandina Island) (Deem et al., 2010). In this study we use a suite of diagnostic procedures, namely gross and microscopic pathology, electron microscopy, and molecular methods to identify the protozoan parasite involved in the fatal infection of the Little Penguins from Western Australia.

2. Materials and methods

2.1. Gross post mortem and histopathology

Penguin Island penguins found dead, either on the island itself or on nearby mainland beaches, by local residents, rangers, officers of state government bodies and researchers are frequently presented to MUVH for post mortem examination; 40 such examinations were carried out in 2012, and 23 in 2013. Under routine post mortem protocols, the penguins were weighed and examined externally for evidence of injuries and ectoparasites. Body condition scores were estimated based on the birds' bodyweight, fullness of pectoral musculature and the presence of subcutaneous and abdominal fat stores. The weights of the liver, spleen and abdominal fat pad were interpreted with reference to a published survey of *E. minor* organ weights compiled from birds from the Otago coast on New Zealand's South Island (Hocken 2000). A range of formalin fixed tissues were retained for routine histopathology. In some cases, impression smears of liver and/or spleen were made and stained with Wright's Giemsa stain.

2.2. Electron microscopy

Samples measuring approximately $1 \times 1 \times 3$ mm were collected from fresh carcasses at the time of post mortem and fixed in glutaraldehyde at 4 °C until required. The glutaraldehyde was removed and replaced with Dalton's chrome osmic acid, then the sample was covered and allowed to fix for approximately 90 min. The sample was washed four times in 70% alcohol, followed by a 5 min wash in 90% alcohol, then a 5 min wash in 95% alcohol and then three washes in absolute ethanol for 3.5 min each. The sample was then washed in two changes of propylene oxide over 10 min before being left in a 60:40 solution of propylene oxide and epoxy resin for 1 h. Finally, the sample was left on a rotator overnight. The following day, the sample was placed in a vacuum chamber, at a negative pressure of approximately 30 inches of mercury, before it was embedded in fresh epoxy resin and baked at 60 °C for 24 h. Thin sections were made at 90 nm thickness on a Reichert Ultracut E ultramicrotome. These sections were collected onto 200 mesh copper grids and stained with lead citrate and uranyl acetate. Grids were examined using a Philips CM100 BioTwin Transmission Electron Microscope (Eindhoven, The Netherlands).

2.3. Polymerase chain reaction and sequencing

Samples from 10 of the affected penguins were investigated by PCR initially at an external laboratory (Center for Evolutionary and Theoretical Immunology, University of New Mexico, Albuquerque, NM, USA), and then later at Murdoch University. Specifically, at the University of New Mexico (UNM), tissue samples from 10 penguins were assayed for *Plasmodium/Haemoproteus* by PCR; these were the first 10 cases diagnosed in 2006, 2008, 2011 and 2012 (Cannell et al., 2013, 2014).

Subsequently at Murdoch University, DNA was extracted from archived tissues from nine of the same penguins that had been tested at UNM. DNA was also extracted from four more penguins that had not been tested at UNM, but that had evidence of the same protozoal infection making a total of 13 cases for which tissues were available. For comparison, DNA was extracted from a further eight penguins that had died of other causes. These 21 samples were tested for *Toxoplasma gondii*.

A *T. gondii* specific nested PCR using primers that amplify an approximately 529bp fragment of the B1 gene (Grigg et al., 2001) was used to detect infection with this parasite. B1 PCR reactions were performed in a total volume of 25 µl containing 0.2 U Taq DNA Polymerase, 200 µM of dNTPs, 0.8 µM of each primer, and 30 ng of DNA template. Amplification was performed in a PT100 thermocycler (MJ Research). The thermal cycle program for both the primary and secondary PCRs

was as follows: the tubes were held at 95 °C for 1 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min. At completion of these cycles the samples were held at 72 °C for a further 5 min, then at 10 °C until removal from the thermocycler.

PCR products were run on a 1.5% agarose gel stained with SYBR safe (Invitrogen, USA), and visualized with a dark reader trans-illuminator (Clare Chemical Research, USA). Bands were cut from the gel, purified using the Agencourt AMPure PCR Purification system (manufacturer's instructions), and sequenced using forward and reverse primers in an ABI Prism™ Terminator Cycle Sequencing kit (Applied Bio-systems, California, USA) on an Applied Bio-system3730DNA Analyser (State Agricultural Biotechnology Centre). Chromatograms derived from both strand sequences were assembled using Geneious Pro R10 software and consensus sequences were compared with reference sequences in GenBank using the nucleotide Basic Local Alignment Search Tool (BLASTN).

PCRs to detect a partial region of the 18S rRNA gene of piroplasmid parasites in the genera *Babesia* and *Theileria* were also carried out using primers as published in Jefferies et al., 2007).

2.4. Immunohistochemistry (IHC)

Toxoplasma IHC was performed on sections from six cases with histological evidence of protozoan infection. Two additional samples with no histological or PCR evidence of protozoan infection were included as negative controls. Toxoplasma IHC was performed using a polyclonal antibody (Thermo Scientific Polyclonal (Goat) PA5-16921). Each slide was washed in water, then treated with 3% Hydrogen Peroxide for 15 min. Slides were washed with distilled water followed by TRIS wash solution, and then treated with normal goat serum for 10 min. The goat serum was poured off and the antibody applied and incubated for 45 min. A set of no primary antibody slides were also prepared omitting the antibody application, but washing with the same buffer solution. All slides were then washed and treated with the secondary antibody reagent for 30 min, after which they were washed and treated with a diaminobenzidine substrate-chromogen solution for 3 min. Slides were counterstained with Harris' Haematoxylin, washed in Scott's tap water substitute, then dehydrated, cleared and coverslipped with DPX.

3. Results

3.1. Gross post mortem and histopathology

In 16 cases that had been provisionally diagnosed with avian malaria from 2011 to 2013, characteristic gross findings were: birds in good body condition with livers tending to be large, moderately to markedly enlarged spleens, and multiple, small, randomly-distributed foci of necrosis in these organs (Figs. 1 and 2). On histology, there were small (approximately 2 μm), ovoid protozoal organisms, occurring free and in cysts, in the necrotic lesions in 15 of 16 livers and 14 of 16 spleens examined (Figs. 3 and 4). Other tissues in which the protozoa were often detected were the heart and lungs, and, much less commonly, adipose tissue and pericardium. The protozoa were also detectable on cytology of splenic impression smears stained with Wright's Giemsa stain (Fig. 5).

Fig. 1. Enlarged liver with multiple, pinpoint cream to white spots.

3.2. Electron microscopy

Figs. 6–8 show the ultrastructural features of the parasite. In Fig. 6 there is a host erythrocyte adjacent to a large, possibly phagocytic, cell. In the cytoplasm of the large cell, abutting its nucleus, there is a cluster of four protozoal organisms (one marked with a P), each approximately 1.5–2 μm across. Though a membrane cannot be seen, the clear space surrounding the parasites is distinctly different from the large cell's cytoplasm, suggesting that the parasites are isolated in a vacuolar space. One organism is oriented to show the typical, slightly elongated apicomplexan morphology with a rounded base, a basal nucleus (N) and a cluster of organelles congregating at the tapered apex (arrow), features that can also be observed in Fig. 7. Fig. 8 shows a parasite in the process of endodyogeny.

These ultrastructural features identify the organism as an apicomplexan, though further identification to the level of genus, or even family, is not possible. At a simplified level, all apicomplexans have both sexual and asexual stages of reproduction. Sexual reproduction involves the fusion of macrogametes to microgametes by a process of syngamy (Schmidt and Roberts 1985), and by definition this occurs in the definitive host, though the macro- and microgametes themselves may have formed in either the definitive host or an intermediate host. Sexual reproduction results in the production of infective sporozoites, which, in the appropriate host tissue, multiply by endopolygeny to produce one or more generations of merozoites. Merozoites divide to form macro- and microgametes for the next round of sexual reproduction. After reproduction, the parasites lyse the host cell and emerge to the extracellular environment whereupon they may infect new cells. Thus, a heavy parasite burden can cause significant host tissue necrosis. In *Toxoplasma,* asexual reproduction occurs by a process of endodyogeny, a special form of endopolygeny in which only two daughter cells are produced, both by incorporating some organelles from the mother cell and by de novo synthesis of others (Nishi et al., 2008). Endodyogeny is not unique to *Toxoplasma*, however, having been reported in the apicomplexan genera *Cytoisospora* (Dubey 2014), *Isospora* (Lindsay et al., 1998), *Sarcocystis* (Paperna 2002) *Besnoitia* (Dubey and Lindsay 2003) and *Neospora* (Sundermann and Estridge 1999). Thus, the evidence of endodyogeny in Fig. 8 is suggestive of, but not definitive for, *Toxoplasma* infection. The morphologic features of the disease (random multifocal necrosis in the liver and spleen) make *Babesia* infection unlikely given that *Babesia* is strictly an erythrocyte parasite in the vertebrate host, and it does not

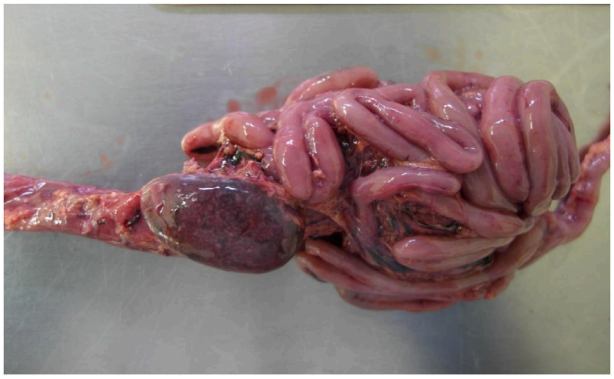

Fig. 2. Enlarged spleen with speckled tan areas of discolouration.

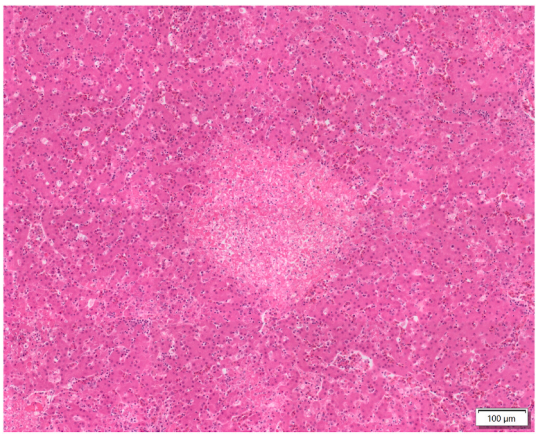

Fig. 3. Liver, necrotic focus (the area of relative pallor, within which numerous organisms were identifiable) (5 µm section, Haematoxylin and Eosin stain, 100x).

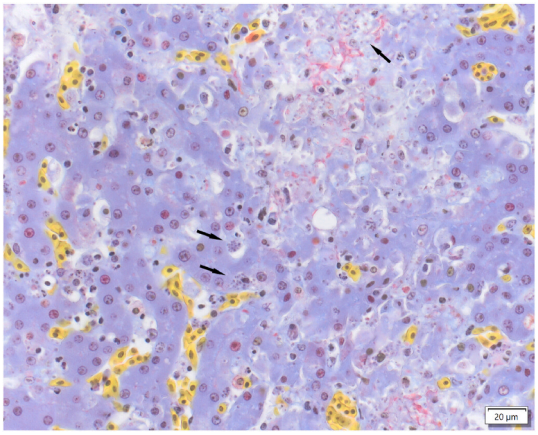

Fig. 4. Liver, intact and necrotic hepatocytes and numerous protozoa (arrows), free and within cysts (5 µm section, Martius Scarlet Blue stain, 400x). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

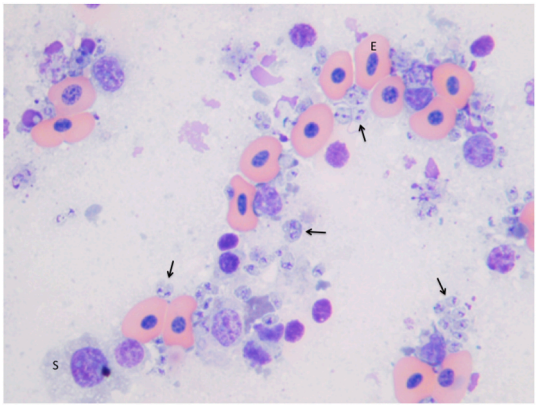

Fig. 5. Splenic impression smear, erythrocytes (E), splenic stromal cells (S) and numerous protozoa (arrows) (Wright's Giemsa stain, 1000x).

replicate in fixed cells in tissues (Homer et al., 2000).

3.3. Polymerase chain reaction and sequencing

PCR assays at MUVH found that 15 samples were positive and 6 were negative for *Toxpolasma*. All DNA extraction blank and PCR negative controls were negative. The 15 positive samples comprised 12 of the 13 cases with gross and microscopic evidence of protozoal infection, and 3 of the 8 comparison cases that had died of other causes. Amplicons from 15 of the penguin samples were sequenced and a 507 bp sequence was obtained from each sample (Fig. 9). All sequences were identified as *Toxoplasma gondii* isolates by BLAST searches (98%–100% similarity; 100% query coverage). Three sequences representing the genetic variants found were submitted to GenBank (accession numbers OM522323–OM522325). As only a single locus (B1) was amplified, it is possible to say that two samples are Archetypal I and 13 samples are either Archetypal II or III; to distinguish between them, additional loci such as GRA6 and APICO would need to be amplified and sequenced. All piroplasmid PCRs were negative.

3.4. Immunohistochemistry (IHC)

In each of the cases that had histopathological evidence of tissue necrosis and protozoan parasites, there was distinct positive staining which was colocalised with the necrotic regions (Fig. 10). In the cases without evidence of parasites, there was no additional staining beyond the background stain seen in the negative control slides. At high power, the uptake of stain was clearly associated with the organisms in the lesions (Fig. 11).

4. Discussion

Initially, the purpose of the work presented here was intended to confirm or refute the provisional diagnosis of avian malaria in the 13 most recent cases. PCR conducted at an external laboratory found no evidence of *Plasmodium*, but did detect the closely related haemoparasite *Haemoproteus* in 4 of 10 sampled penguins. However, all 10 had histologically visible parasites in liver and spleen sections, so if the PCR was detecting these organisms, it is not clear why 6 of 10 were negative. Also, of those that were positive, PCR amplifications had been repeated on up to 3 occasions before a visible band was detected by gel electrophoresis, indicating limited sensitivity for this assay (Cannell et al., 2013). By this technique, the detection of *Haemoproteus* DNA was not well correlated with the presence of parasites in the tissues. Also, extra-erythrocytic stages of *Haemoproteus* (meronts and megalomeronts) frequently form much larger cystic structures (Valkiūnas and Iezhova, 2017) than were seen in these sections, which is a useful morphological indicator to reduce suspicion of haemoproteosis. Extra-erythrocytic stages of *Plasmodium* species are morphologically variable (Valkiūnas and Iezhova, 2017), but can be consistent with the smaller cysts seen in these tissues, so additional diagnostic methods beyond histopathology (including PCR, immunohistochemistry and/or in situ hybridization (Dinhopl et al., 2011; Himmel et al., 2019)) are required for definitive diagnosis.

Toxoplasma was detected by PCR in 12 of 13 penguins diagnosed with protozoal infections, and in 3 of 8 penguins diagnosed with other causes of death. On the sum of this evidence, the fatal protozoal infections are considered to be toxoplasmosis, but PCR findings suggest that some of the penguins were, or had been, also host to *Haemoproteus* parasites. Given the apparent limited sensitivity of the PCR assay implemented against this organism during the present study, it cannot be stated with confidence how many birds were truly infected. In general, *Haemoproteus* infections in birds are quite common and usually cause mild or no clinical disease (though there are exceptions, especially when infection occurs in aberrant hosts). The presence of *Toxoplasma*, however, is strongly correlated with disease in these birds, and immunohistochemistry positively identifies the parasites within the lesions of 6 out of 6 of the affected birds, including the single case that was positive by light microscopy but negative by PCR, suggesting that the PCR result represents a false negative. The gross, histological, ultrastructural, immunohistochemical and PCR results are all consistent with fulminant

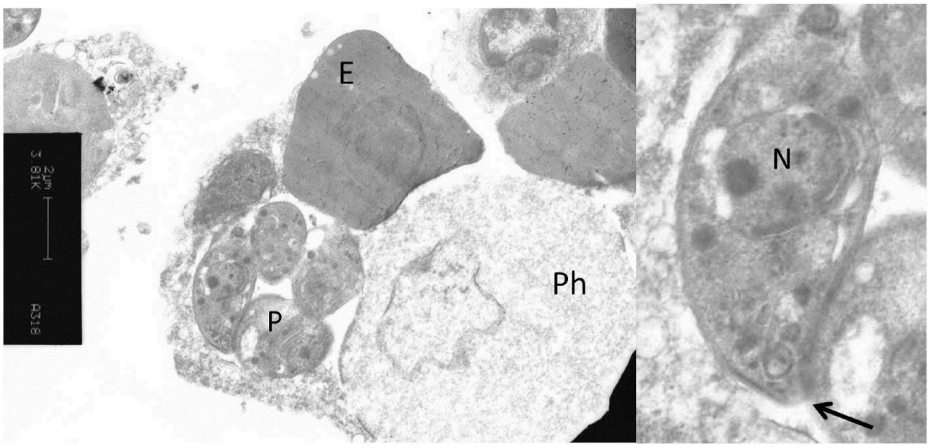

Fig. 6. (And detail) – four parasites in a cyst within the cytoplasm of a host cell, spleen (x3810, bar = 2 μm) Image left: E - erythrocyte, Ph - phagocyte, P - protozoa; image right (detail): N - nucleus.

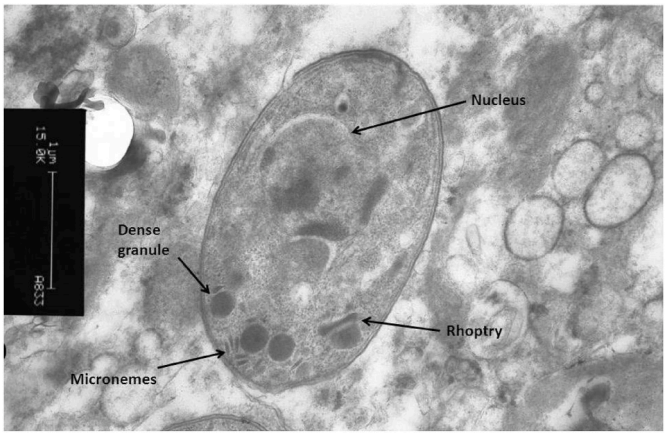

Fig. 7. Protozoan with apical complex (x15000, bar = 1 μm).

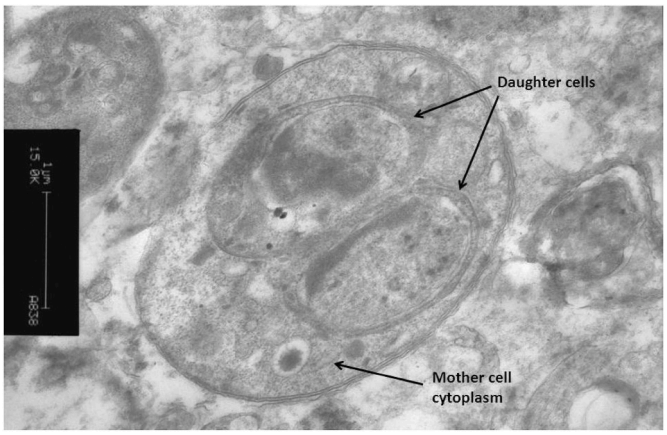

Fig. 8. Dividing protozoan forming merozoites (x15000, bar = 1 μm).

toxoplasmosis being the cause of death in these penguins.

The pathological changes observed are in agreement with a previous published case of toxoplasmosis in one Little Penguin (Mason et al., 1991), and are also consistent with the pathology reported in experimental toxoplasmosis in other animal species (such as eastern barred bandicoots (Bettiol et al., 2000)) though it is worth noting that *Toxoplasma* tachyzoites and bradyzoites are widely distributed in body tissues, and the range of lesions which may occur when infections progress beyond the acute stage is extensive (Canfield et al., 1990). *Toxoplasma* is a global parasite, which very commonly infects Australian wildlife species (Pan et al., 2012; Parameswaran et al. 2009, 2010, Wendte et al., 2011), and which causes fatal infections in wild birds (Dubey et al., 2001, Ferreira et al., 2012; Las and Shivaprasad, 2008; Howe et al., 2014). In distinction to the life cycle of apicomplexan haemoparasites, *Toxoplasma* requires no arthropod vector. The definitive host is the cat, and transmission of disease may occur by ingestion of infective oocysts from cat faeces, or by ingestion of tissue cysts in animal tissues (as well as, in all likelihood, by vertical transmission in utero (Thompson 2013). Because sexual development only occurs in cats and it is not transmitted by a vector, it might be expected that exposure to *Toxoplasma* would be low in marine wildlife; note that cats are not present on Penguin Island, which is located 600m offshore from the city of Rockingham. However, it is also emerging that *Toxoplasma* infection is not uncommon in many marine mammals, implying that the disease can be transmitted by contamination of waterways with cat faeces (Jones and Dubey 2010). Furthermore, it has been shown that filter-feeding fish will take up *Toxoplasma* oocysts from contaminated water, and that mice can become infected when fed on these fish (Mason et al., 1991). Notably, one species of fish that has been demonstrated to take up *Toxoplasma* cysts and transmit the disease is the pilchard (*Sardinops sagax*) (Mason et al., 1991), an important prey species for the Little Penguins of Penguin Island (Klomp and Wooller 1991). Also, it has been shown that *Toxoplasma* can aggregate on extracellular polymeric substances in marine environments, enhancing the efficiency with which the organism could enter the marine food chain in coastal ecosystems (Shapiro et al., 2014).

Toxoplasma DNA was detected in three out of eight penguins that did not have any of the gross or microscopic lesions associated with severe protozoal disease, implying that infection with *Toxoplasma* is not uniformly fatal in the penguins, and that virulence, dosage and/or comorbidity factors are likely to play a role in determining the outcome of infection in any given bird. Notably, a feature of fatal disease is that the birds were, by and large, in good body condition. It is possible that these penguins were able to gather adequate prey in the local environment without the need to travel far afield, and that the fish they were preying on in that environment themselves carried a large parasite burden, though to prove this would require the testing of local penguin prey species for *Toxoplasma*, which was beyond the scope of this study. Whatever the case, the incidence of toxoplasmosis is most likely dependent on the overall number and the disease status of cats contributing to faecal contamination of local waterways. A serological survey of the penguins would provide a useful indication of the prevalence of exposure in the population.

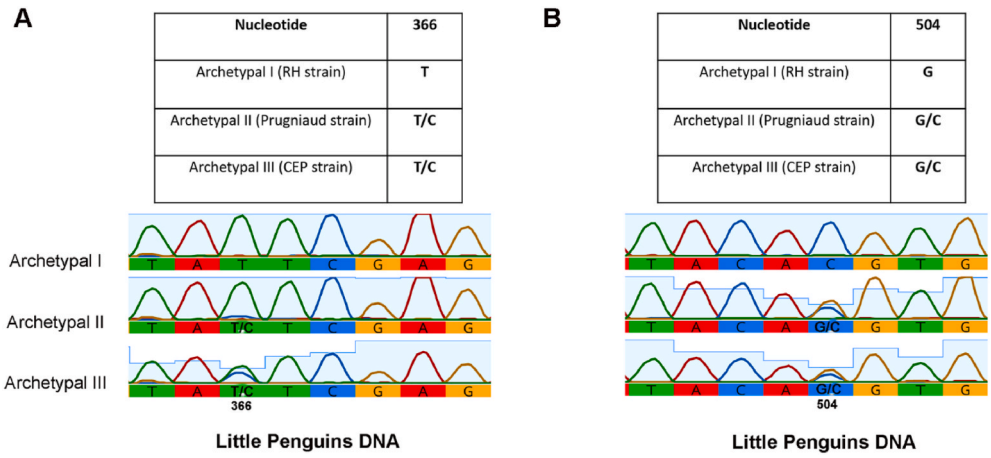

Fig. 9. Similarity of *Toxoplasma gondii* from Little Penguins with representative strains from Archetypals I, II, and III in the B1 gene. A: shows polymorphisms at the 366 nucleotide. B: shows polymorphisms at the 504 nucleotide.

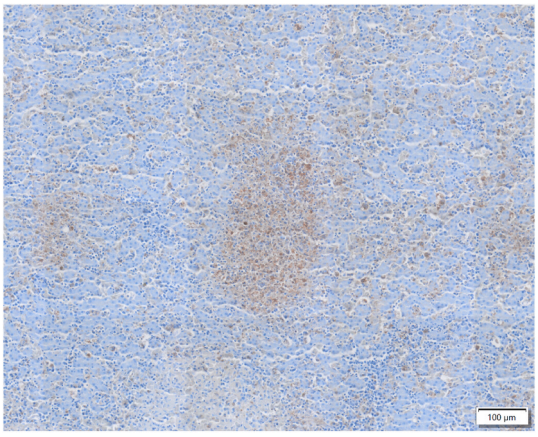

Fig. 10. Liver, several foci of brown staining indicate *Toxoplasma* antigen within a necrotic focus (x100, *Toxoplasma* polyclonal antibody IHC). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

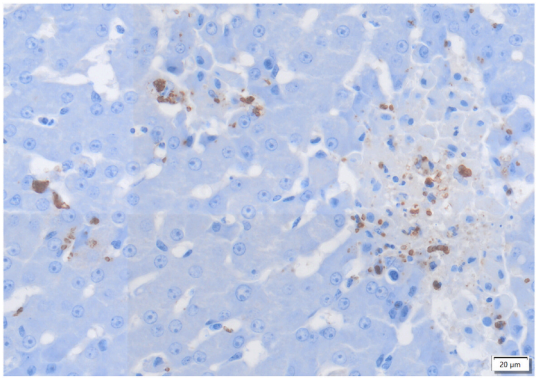

Fig. 11. Liver, staining of protozoan antigen in intact and necrotic hepatocytes (x400 *Toxoplasma* polyclonal antibody IHC).

Declaration of competing interest

None.

Acknowledgements

this study was supported by Murdoch University's Research Masters with Training Program for veterinary residents.

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