Molecular identification of *Sarcocystis wobeseri*-like parasites in a new intermediate host species, the white-tailed sea eagle *(Haliaeetus albicilla)*

Shadbolt, T[1]*, Pocknell, A[2], Sainsbury, AW[1], Egerton-Read, S[3] & Blake, DP[4].

[1] Institute of Zoology, Zoological Society of London, London NW1 4RY, UK

[2] Finn Pathologists, Weybread Suffolk IP21 5TT, UK

[3] Forestry England, South Forest District, The Queen's House, Lyndhurst, Hampshire, SO43 7NH, UK

[4] Royal Veterinary College, Hatfield AL9 7TA, UK

* Corresponding author: Tammy.Shadbolt@ioz.ac.uk

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Abstract

A reintroduced white-tailed sea eagle (*Haliaeetus albicilla*) in moderate body condition was found dead and submitted for post-mortem examination. There were no signs of disease on gross pathological examination. Histopathological examination however revealed the presence of encysted protozoan parasites in pectoral and cardiac muscle sections. Polymerase chain reaction amplification of extracted genomic DNA and sequencing of four regions: the 18S rDNA, 28S rDNA, internal transcribed spacer (ITS) 1 and RNA polymerase B (rpoB) loci, confirmed the presence of a *Sarcocystis* species in pectoral and cardiac muscle which appeared phylogenetically similar to *Sarcocystis wobeseri*. This is the first report of *S. wobeseri*-like infection in a white-tailed sea eagle revealing a new intermediate host species for this parasite.

Introduction

Within the UK the white-tailed sea eagle is protected under the Wildlife and Countryside Act 1981 and The Nature Conservation (Scotland) Act 2004, and is included on the Red List of UK birds of conservation concern. The white-tailed sea eagle examined here was one of six birds reintroduced under licence from Natural England onto the Isle of Wight as part of a conservation initiative established by the Roy Dennis Wildlife Foundation and Forestry England. The birds were collected as juveniles from nests in Scotland under licence, issued by Scottish Natural Heritage, in June 2019 and translocated to the Isle of Wight where they were held at a protected location prior to release in August 2019.

Protozoans of the genus *Sarcocystis* are intracellular parasites which infect a wide range of taxa including mammals, birds and reptiles, and have a global distribution (Kirillova et al. 2018). *Sarcocystis* spp. have an

obligatory two-host life cycle. Herbivorous and omnivorous animals usually acquire *Sarcocystis* spp. infection through the ingestion of oocysts-sporocysts in faecally contaminated water or food and sarcocysts are formed in the muscle tissues of these intermediate hosts. Definitive hosts most commonly acquire *Sarcocystis* spp. infection through the ingestion of infected intermediate prey species through scavenging or predation. Oocysts are formed in the intestinal mucosa of these definitive hosts and sporulate prior to excretion (Gjerde et al. 2018). A large number of *Sarcocystis* spp. can infect birds, with *Sarcocystis falcatula* and *Sarcocystis calchasi* recognised as two of the most pathogenic (Dubey et al. 2016). Several *Sarcocystis falcatula*-like spp. have been described and *Sarcocystis falcatula* is considered to most likely constitute a complex of several species. Opossum species (*Didelphidae virginiana* and *Didelphidae albiventris*) are the natural definitive hosts and numerous birds including raptors are considered intermediate hosts within the Americas (Box and Duszynski 1978; Dubey et al. 2016). *Sarcocystis calchasi* is of importance within Europe as well as the Americas and two raptor species, the Northern goshawk (*Accipiter g. gentilis*) and European sparrowhawk (*Accipiter nisus*), act as definitive hosts for this parasite with pigeons and psittacine birds considered intermediate hosts (Olias et al. 2010; Dubey et al. 2016). The definitive host of *Sarcocystis wobeseri* is unknown. Microscopic, thin walled sarcocysts have however been described and identified as *Sarcocystis wobeseri*, based on polymerase chain reaction (PCR) DNA amplification and sequencing of the 18S rDNA, 28S rDNA, and internal transcribed spacer (ITS) 1 regions, in three avian intermediate hosts: the barnacle goose (*Branta leucopsis*), mallard duck (*Anas platyrhynchos*) and herring gull (*Larus argenticus*) (Kutkienė et al. 2010; Prakas et al. 2011, 2020). To the best of our knowledge *Sarcocystis wobeseri* has not previously been detected in a raptor acting as an intermediate host. In this study the submission of a white-tailed sea eagle found dead for post-mortem examination revealed a *Sarcocystis wobeseri*-like infection in pectoral and cardiac muscle and we report the investigation here to increase our understanding of the host range and phylogenetics of this parasite, specifically in a new intermediate host species.

Materials and methods

Case history

A first-year male white-tailed sea eagle, satellite harnessed and radio tracked, was released on 22nd August 2019 and found dead next to a hedge line on the west coast of the Isle of Wight, UK on 1st October 2019. It was known that the bird had been active in the month before death and seen feeding on the carcase of a porpoise. Tracking

data suggested that the bird had become inactive two days prior to being found dead. A post-mortem examination was carried out at the Institute of Zoology, Zoological Society of London on 3rd October 2019 according to a standardised avian post-mortem examination protocol (Molenaar 2008). Radiographs were taken prior to examination of the carcase. Tissue samples of pectoral muscle, thyroid, heart, lung, liver, spleen, kidney, adrenal gland, oesophagus, proventriculus, ventriculus, small intestine, large intestine and brain were taken into 10% buffered formalin for histopathology. Samples were taken for parasitology, bacteriology, genetic studies and archiving, but most results are not reported here because they are not relevant to the purpose of the paper.

Histopathology

Approximately 15 mm^3 samples of tissues as listed above were fixed in 10% neutral buffered formalin, sectioned at 0.5 µm and prepared for histological examination with haematoxylin and eosin (HE) stain. Sections of skeletal muscle and heart muscle were subsequently examined with Gram-Twort, Ziehl-Neelsen, and Perls' Prussian Blue stains. Standard histological techniques were employed (Bancroft 2008).

Molecular identification and characterisation

Approximately 25 mg of pectoral and cardiac muscles were excised from the centre of samples collected and frozen at -20ºC at the time of post mortem. Total genomic DNA was extracted using a DNeasy Blood and Tissue kit as described by the manufacturer (Qiagen, Hilden, Germany).

Four PCR assays were performed using primers targeting the 18S and 28S ribosomal DNA (rDNA), internal transcribed spacer 1 (ITS1) and RNA polymerase B (rpoB) sequences. Each 50 µL PCR reaction contained 2.0 µL of DNA, 0.2 µL of each respective forward and reverse primer (100 µM/ml; synthesised by Sigma, Gillingham, UK; Table 1), 22.6 µL of molecular grade water and 25 µL of MyTaq Mix (2×) (Bioline, Nottingham, UK). All PCR cycles followed a standard protocol: initial denaturation for 1 min at 95°C, followed by 30 cycles of 0.5 min at 94°C, 1 min annealing (as shown in Table 1) and 1.5 mins at 72°C, with a final elongation phase of 72°C for 7 mins. No template negative controls were run in parallel for each assay using molecular grade water in place of DNA.

PCR products were resolved by agarose gel electrophoresis using 1% (w/v) Ultrapure™ Agarose (Thermo Fisher Scientific, Leicester, UK) prepared in 1× Tris-borate-EDTA buffer with 0.01% (v/v) SafeView Nucleic Acid Stain (NBS Biologicals Ltd., Cambridgeshire, UK). Amplicons of the anticipated size (Table 1) were purified using a QIAquick PCR Purification Kit (Qiagen) and sent for Sanger sequencing using the same primers employed in the original amplification (GATC Biotech AG, Konstanz, Germany). Sequences were manually curated and assembled using default parameters with CLC Main Workbench (version 8.0.1), and annotated following comparison using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were aligned with panels of published reference sequences using CLC and exported to MEGA X (Kumar et al. 2018). Optimal phylogenetic models were identified using Akaike's Information Criterion (AIC). The Maximum Likelihood (ML), Neighbor Joining (NJ) and Maximum Parsimony (MP) methods were used to estimate sequence phylogeny, all with 1,000 bootstrap iterations. *Toxoplasma gondii* was used as an out-group.

Table 1: Forward and reverse primers used for PCR amplification from *Sarcocystis* spp. DNA. GenBank accession numbers for the reference *S. wobeseri* sequences are shown. *F suffix = forward primer, R = reverse primer.

Locus	Primer name*	Primer sequence (5′-3′)	Annealing °C (amplicon size)	Reference	*S. wobeseri* reference
18S rDNA	SarAF SarAR	CTGGTTGATCCTGCCAGTAG TTCCCATCATTCCAATCACT	56 (~1,500 bp)	(Kutkienė et al. 2010)	GQ922886
28S rDNA	KL-P1F KL-P2R	TACCCGCTGAACTTAAGCAT TGCTACTACCACCAAGATCTGC	58 (~1,468 bp)	(Kutkienė et al. 2010)	GQ922887
ITS1	P-ITSF P-ITSR	ATTGAGTGTTCCGGTGAATTA GCCATTTGCGTTCAGAAATC	56 (~940 bp)	(Kutkienė et al. 2010)	MN450369
rpoB	RPObF RPObR	TAGTACATTAGAAATCCCTAAAC TCWGTATAAGGTCCTGTAGTTC	52 (~762 bp)	(Wendte et al. 2010)	MH138325

Results

Gross pathological and histopathological findings

At post-mortem examination this white-tailed sea eagle weighed 2798g and was in moderate body condition with no subcutaneous fat. The oesophagus, proventriculus, ventriculus and intestinal tract were empty of content. There was a rupture in the left axilla infested with fly larvae but without inflammation. There was extensive green discolouration of the coelomic viscera. There were no other significant findings on gross post-mortem examination. Bacteriology and toxicology tests did not reveal a diagnosis and are not reported here because they are not relevant to the purpose of the paper.

Histopathological examination of sections of pectoral muscle revealed six myocytes greatly expanded by encysted oval protozoan parasites consistent with sarcocysts, which ranged between 50-60 µm in diameter and up to 300 µm in length. The sarcocysts had a thin and seemingly smooth wall of approximately 1.1 µm thickness. The cysts

were densely packed with small (5 to 7 µm long × 1.2 µm wide) lancet- or banana-shaped bradyzoites (Figure 1). No inflammation or necrosis was visible. A Perls' Prussian Blue stain slightly highlighted the bradyzoites within the cysts. A Gram-Twort stain showed the bradyzoites in pink. Ziehl-Neelsen stains showed the bradyzoites in blue (not acid-fast). Examination of a section of striated heart muscle revealed a single sarcocyst similar to the above which measured 30 µm in diameter and 83 µm in length. Whether this parasite was truly the same species could not be determined by histopathology alone. There was no associated inflammation or necrosis. Sections of intestine were examined. No intestinal parasitic lifestages were visible.

Molecular identification and characterisation

Single amplicons of the estimated size were resolved for all four PCR assays using genomic DNA extracted from pectoral and cardiac muscles. Identical sequences were generated for each assay from both muscle sources. Sequence analysis using BLASTn identified greatest similarity for all amplicons with reference sequences derived previously from *S. wobeseri*. Sequence identity for the 18S rDNA and rpoB amplicons was 100%, with 97 and 96% coverage of accession numbers GQ922886 and MH138325, respectively, and no gaps. Sequence identity for the 28S rDNA amplicons was 99%, with 99% coverage of reference sequence GQ922887 and no gaps. Sequence identity for the ITS1 amplicons was 99%, and while coverage of reference sequence MN450369 was lower (85%), no gaps were detected. Sequences derived here have been deposited in the European Nucleotide Archive (ENA) under the study accession PRJEB40290 with individual accession numbers LR884238-LR884241.

The 18S and 28S rDNA, ITS1 and rpoB sequences produced here were aligned with reference sequences downloaded from GenBank representing between 13 and 18 *Sarcocystis* spp. for each, as well as *T. gondii* as an outgroup for the rDNA sequences (Online Resource Figure 1, Online Resource Table 1). ML, NJ and MP phylogenies presented consistent topologies for each sequence dataset, so only ML is presented here. The optimal models used were K2+G (18S rDNA), HKY+G+I (28S rDNA and ITS1) and T92+G (*rpoB*). The 18S and 28S rDNA sequence alignments were highly conserved, consistently identifying *S. wobeseri* as the closest relative species, although consensus support was low (Online Resource Figure 1 a, b). The ITS1 and rpoB sequence alignments were more discriminatory within the *Sarcocystis* genus, again identifying *S. wobeseri* as a close relative although consensus support was low for many branches (Online Resource Figure 1 c, d). The optimal

model for the concatenated sequence alignment was HKY+G. Consideration of the phylogeny inferred from the concatenated sequence alignment supported annotation of the sample as *S. wobeseri*-like (Figure 2).

Discussion

In this case a reintroduced white-tailed sea eagle was found dead after a short period of inactivity. Examination of the gastrointestinal tract suggested that it had not eaten for days and was dehydrated. It was concluded this bird was unable to acquire food and likely was sick in the days before death. The green discolouration throughout the coelom was considered to be pseudo-melanosis. The absence of inflammation and presence of fly larvae in the left axilla suggested this lesion occurred after death. Pathological examination did not reveal evidence of disease. The encysted apicomplexan parasites seen on histopathology of pectoral and cardiac muscle sections were morphologically consistent with *Sarcocystis* spp.. In this bird no lesions were visible in association with any of the sarcocysts so the clinical significance of the infection is uncertain. No intestinal parasitic lifestages were visible but the sections were severely autolysed so infectious agents or other lesions could easily have been obscured. Molecular techniques were used to identify the *Sarcocystis* spp. in the sections of pectoral and cardiac muscle as similar to *S. wobeseri*. The low levels of consensus support for relationships between some species when compared using single genomic loci reinforces identification as *S. wobeseri*-like, with greatest support from comparison of concatenated sequences.

At least 12 species of *Sarcocystis* are thought to use birds as definitive hosts and at least 25 species are thought to use birds as intermediate hosts (Atkinson et al. 2008; Prakas et al. 2020). *Sarcocystis* infection has previously been reported in the white-tailed sea eagle. Gjerde et al. (2018) detected *Sarcocystis halieti* n. spp., *Sarcocystis lari* and *Sarcocystis truncata* oocysts in the small intestine of a white-tailed sea eagle suggesting the white-tailed sea eagle was a definitive host for all three *Sarcocystis* spp. Unidentified sarcocysts were additionally found in the cardiac muscle of the same eagle and therefore the white-tailed sea eagle was also an intermediate host. To the best of our knowledge, *S. wobeseri*-like parasites are described from a white-tailed sea eagle for the first time in our study. Here the discovery of sarcocysts in the pectoral and cardiac muscle suggested *S. wobeseri*-like parasites were using the white-tailed sea eagle as an intermediate host. As an intermediate host, this white-tailed sea eagle most likely acquired infection via a faecal-oral route through the accidental ingestion of water contaminated with oocysts-sporocysts.

An understanding of the pathogenesis of sarcocystosis in birds has been gained from research on *Sarcocystis falcatula* and *Sarcocystis calchasi*. Disease associated with *Sarcocystis falcatula* infection in old world psittacines and experimentally infected passerines can be attributed to the presence of meronts during early infective phases (Atkinson et al. 2008). Meronts form in the lungs and liver by day two, the first sarcocysts form in the cardiac muscle by day seven and pectoral muscle by day eight (Atkinson et al. 2008). Pulmonary and hepatic disease manifests as acute anorexia, weakness, difficulty breathing and neurological signs in these intermediate hosts (Hillyer et al. 1991; Dubey et al. 2016). Disease associated with *Sarcocystis calchasi* infection in pigeons and psittacine birds is attributed to schizogonic development in neural cells (Dubey et al. 2016). Disease in these intermediate hosts manifests as meningoencephalitis and neurological signs such as depression, trembling and paralysis (Olias et al. 2009). Whilst infection with pathogenic *Sarcocystis* spp. represents a concern, especially in species of conservation interest, infection of avian intermediate hosts with other *Sarcocystis* spp. appears rarely to cause clinical signs even when infection intensity is high (Atkinson et al. 2008). Dohlen et al. (2019) conducted a study in the southeastern United States to determine the prevalence of *Sarcocystis* spp. sarcocysts in the muscles of raptors acting as intermediate hosts. Sarcocysts were identified in the pectoral muscle of 39 out of 204 raptors and in the heart muscle of nine birds which also had sarcocysts in the pectoral muscle. Research however found no significant association between the presence of sarcocysts in raptor pectoral or cardiac muscle and a diagnosis of disease (Dohlen et al. 2019). In this white-tailed eagle no association could be demonstrated between the presence of *S. wobeseri*-like sarcocysts in the pectoral and cardiac muscle tissues of the bird and disease.

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