Sarcocystis rileyi in UK free-living wildfowl (Anatidae): surveillance, histopathology and first molecular characterisation

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Abstract

Reports from UK hunters of unusual findings of 'rice-like grains' in muscles of shot wildfowl (Anatidae) coincided temporally with finding of sarcocystosis in a small number of ducks found as part of the Wildfowl & Wetlands Trust long term general surveillance of found dead waterbirds. Wildfowl are known to be the intermediate hosts of at least five species of coccidian parasites of the genus *Sarcocystis*, with the presence of *Sarcocystis rileyi* being relatively recently confirmed in wildfowl in northern and eastern Europe.

This study utilises four approaches to investigate *Sarcocystis* spp. infection in UK wildfowl: firstly through a hunter questionnaire that captured case data over historical shooting seasons; secondly through an online reporting system, thirdly DNA sequencing to characterise UK cases; and fourthly histological myopathy assessment of infected wildfowl pectoral muscle.

Our questionnaire results suggest that *Sarcocystis* infection is widely distributed throughout the UK, with infection observed in 10 Anatidae species and reported cases increasing annually since the 2010/11 shooting season, with the online reporting system reflecting this increase. DNA sequencing of the 18S rRNA gene and internal transcribed spacer (ITS)-1 region from UK isolates confirmed the presence of *S. rileyi* in the five species of dabbling duck tested and myopathy associated with these infections is described. Further research is required to ascertain the impacts *S. rileyi* infection has on host health, fitness and survival of these migratory species.

Keywords

Anatidae, emerging disease, rice breast disease, *Sarcocystis*, sarcocystosis, wildfowl

Introduction

Sarcocystis species (Apicomplexa: Sarcocystidae) are intracellular cyst forming protist parasites of mammals, birds, reptiles and fish (1). Anatidae are the intermediate hosts of at least five *Sarcocystis* species (2,3), with the asexual stage of the parasite's lifecycle (schizogony) occurring within the vascular endothelium and subsequent sarcocyst development in the skeletal and occasionally cardiac muscles (4). *Sarcocystis rileyi* is the most commonly reported *Sarcocystis* sp. in North American wildfowl, frequently utilising the northern shoveler (*Anas clypeata*) and other *Anas* dabbling duck species as an intermediate host, and the striped skunk (*Mephitis mephitis*) as a definitive host (5–7).

Wildfowl *Sarcocystis* infection has previously not been associated with increased mortality, however investigations into health impacts have been limited (4). Granulomatous myositis, associated with sarcocyst degeneration, has been diagnosed in northern shoveler and mallard (*A. platyrhynchos)* (8). Such conditions could potentially predispose infected individuals to reduced fitness and increased predation and shooting risk (7).

Kalisinska et al. (2003) (9) provide a historical perspective of possible *S. rileyi* reports in mainly eastern Eurasia since 1940 with light microscopy confirming the species in mallard in Lithuania in 2003. Since *Sarcocystis* species can share the same microscopic cyst morphology, DNA sequencing provides the most specific test for *S. rileyi* characterisation and typically focuses upon two ribosomal RNA loci, the 18S rRNA gene and the first internal transcribed spacer region (ITS-1). These methods were used to confirm

European *S. rileyi* infection in a mallard shot in 2010 in Lithuania (10). Further DNA-confirmed cases have been reported in 2014 from Lithuania and Finland in mallard, common teal (*A. crecca*) and Eurasian wigeon (*Mareca penelope*), and common eider (*Somateria mollissima*) in Norway (3,11). Potential definitive hosts were suggested in 2015 in Lithuania following molecular identification of *S rileyi* oocysts/sporocysts in hunted red fox (*Vulpes vulpes*) and the introduced raccoon dog(12) (*Nyctereutes procyonoides*).

The hunting community can and do play an important role in wildlife disease study through the detection and reporting of disease events, alongside sample provision for surveillance and research (13). Given the striking lesions, which can be readily identified by those interfacing with wildlife during hunting activities and meat preparation, 'rice-breast disease' (as it is called in hunting communities) in wildfowl offered an opportunity for targeted surveillance. As a result, the wildfowling community were encouraged to play an important role in the study of this potentially emerging disease.

The overall aims of this study were to determine the possible UK emergence of *S. rileyi* in free-living wildfowl and assess the potential impacts parasitic infection may be having on host muscle health. Specifically, the objectives were:

- To assess UK wildfowler experiences of 'rice-breast disease' emergence in the field through a hunter questionnaire, capturing case data over historical shooting seasons, together with an online reporting system during the shooting seasons from 2015/16 to 2018/19.
- To confirm, using DNA sequencing, the presence of *S. rileyi* in hunter-submitted infected wildfowl and investigate the phylogeny of UK isolates.
- To histologically assess the pectoral muscles of the aforementioned wildfowl for sarcocyst-associated myopathy.

Materials and Methods

The UK Wildfowl Sarcocystis Survey is a collaborative project between the Wildfowl & Wetlands Trust (WWT), British Association for Shooting and Conservation (BASC) and University of Liverpool, including a reporting website (www.sarcocystissurvey.org.uk) for suspected wildfowl *S. rileyi* cases. For both the hunter questionnaire and the online reporting system it was accepted that diagnosis in these instances is being made in the field from the observation of the characteristic rice-grain lesions within musculature, rather than laboratory or expert analysis.

Awareness of the project and sarcocystosis was raised through BASC member newsletters and articles within the UK shooting press, which encouraged reporting of cases and submission of affected carcasses or pectoral musculature from the 2015/16 shooting seasons onwards.

Online reporting system

Set up prior to the 2015 winter shooting season, an online reporting system was developed for the wildfowling community to submit new suspected cases of *S. rileyi* in shot UK wildfowl quarry species, accessible through the UK Wildfowl Sarcocystis Survey website (http://www.sarcocystissurvey.org.uk/survey-form/).

Awareness of the online reporting system was raised as discussed above. Respondents were asked to submit one online reporting form per suspected *S. rileyi* case with the following case information: host species, host age and gender (if known), geographic location and shooting season; alongside a simple body condition assessment of the carcase (Good - with some fat, plump breast muscle; Fair - plump breast muscle - no fat; Thin - no fat, some loss of breast muscle (keel showing); or Emaciated - no fat, severe loss of breast muscle (no keel)). Where possible, respondents were encouraged to accompany online reports with photographs of bird carcasses displaying rice-breast disease lesions.

Online reporting forms for quarry shot between the 2014/15 and 2018/19 shooting seasons were used for this study. Forms which were missing information on quarry species or date shot were not included in the analysis. Submissions for quarry shot outside the UK, or for non-wildfowl species, were also removed prior to analysis. Data were anonymised and a Pearson's chi-squared test performed to determine whether the observed number of shot individuals per quarry species differed from expected values, as determined by the National Gamebag Census (14).

Hunter questionnaire

In 2017 an anonymous, online questionnaire was designed using the online survey software SurveyMonkey (www.surveymonkey.net) to assess BASC member historical experiences of rice-grain lesions in legally shot UK wildfowl quarry species over the past 12 shooting seasons, with cut-offs based upon the first publication of European wildfowl *Sarcocystis* research in Poland and Lithuania (9,15).

Questions were incorporated for respondents who had and who had not encountered the infection. Case information was the same as the online reporting system. To assess hunter harvest effort, the questionnaire included an approximation of total annual bag of ducks and geese over the past seven seasons, alongside a self-evaluation as to whether that individual's annual bag was increasing, decreasing or remaining constant across recent years.

Hunters were asked whether they thought sarcocystosis in UK wildfowl represented an emerging infection as defined by the OIE, i.e. a "new occurrence in an animal of a disease, infection or infestation […] resulting from: a) a change of a known pathogenic agent or its spread to a new geographic area or species; or b) a previously unrecognised pathogenic agent" (16).

The questionnaire web-link was distributed to 106,485 BASC members via email and also uploaded onto the BASC Facebook page. Respondents had a four-week period, from late June to mid July 2017, to reply. Statistical analysis was undertaken in R-Studio Version 1.0.153, employing the Fisher's exact, Mann-Whitney U and Spearman's correlation tests, all with significance set as $p<0.05$.

It is accepted that reported cases from the 2015/16 season would potentially overlap with reports submitted online.

Molecular identification

Samples

Following contact via the UK Wildfowl Sarcocystis Survey website, 14 samples from 13 hunter-shot ducks were received over the winter 2015/16 and winter 2016/17 shooting seasons, all displaying pectoral macroscopic rice-grain lesions (supplementary Fig. 1) and originating from five dabbling duck species: seven mallard, three Eurasian wigeon, one gadwall (*Mareca strepera*), one northern pintail (*Anas acuta*) and one common teal. Samples originated from widespread UK locations including the Scottish Highlands, County Fermanagh, Cumbria and Gloucestershire. Samples were stored at -20°C prior to defrosting and sarcocyst dissection. Two samples (3 and 4) were submitted in formalin, known to cause significant DNA cross-linking which reduces PCR accessibility (17). Sample 14 had likely suffered significant nucleic acid degradation from autolysis and repeated freeze and thaw cycles. While these samples were inappropriate for molecular analysis, they were retained to increase overall sample numbers for the other analyses.

Genomic DNA extraction

Approximately 25mg of macroscopic sarcocysts were dissected from the pectoral muscles of each submitted duck into sterile 1.5ml Eppendorf tubes. Subsequently, total DNA was extracted from each sample using a DNeasy Blood and Tissue kit as described by the manufacturer (Qiagen UK).

Polymerase chain reaction and amplicon resolution

Three PCR assays were performed for each sample. Initially, diagnosis was confirmed using primers ERIB1 and ERIB10, targeting the coccidian 18S ribosomal RNA subunit (18) (sTable 1). Subsequently, primers specific for the *Sarcocystis* 18S rRNA gene and the 18S rRNA + 5.8S rRNA genes, flanking the first internal transcribed spacer (ITS-1) region, were used to generate amplicons for sequencing and phylogenetic comparison (10). Each 25µL PCR reaction contained 2.0µL of sarcocyst DNA, 0.1µL of each forward and reverse primer (100µM/ml), 10.3µL of molecular grade water and 12.5µL of MyTaq Mix (2×) (Bioline, UK).

All PCR cycles followed the same protocol, with an initial denaturation for 1 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 56°C and 1.5 mins at 72°C, with a final elongation phase for 10 min at 72°C. Negative controls containing molecular grade water in place of sample DNA were used in each PCR assay.

PCR products were resolved by agarose gel electrophoresis using 1% (w/v) Ultrapure™ Agarose (Thermo Fisher Scientific UK Ltd) prepared in 1× Tris-borate-EDTA buffer with 0.01% (v/v) SafeView Nucleic Acid Stain (NBS Biologicals Ltd., Cambridgeshire, UK).

PCR amplicon purification, sequencing and analysis

Amplicons from the 18S rRNA gene and ITS-1 assays of the anticipated size were purified using a QIAquick PCR Purification Kit (Qiagen, UK) 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 6.0.2) and annotated using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). ITS-1 sequences were aligned with a panel of published reference sequences using ClustalW and exported to MEGA 6.06, where the optimal phylogenetic model was identified using the best Bayesian Information Criterion (BIC). 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.

Histopathology of *Sarcocystis* infected pectoral muscle

Submitted pectoral muscle samples were defrosted in containers of 10% neutral buffered formalin (CellPath Ltd, UK). Areas of pectoral muscle with the highest density of macroscopic rice-grain lesions were selected with 1cm x 1cm transverse and longitudinal sections being cut when sample quality allowed. Sections were processed routinely and embedded in paraffin with 5µm diameter sections stained with Haematoxylin and Eosin, prior to histopathological examination.

Each sample was evaluated for the total number of rice-grain lesions present both grossly and any additional microscopic myopathy; comprising inflammation (surrounding sarcocyst and/or distant from sarcocysts), muscle fibrosis and myofibre atrophy. Each of these histological myopathy features was scored out of three by a veterinary histopathologist. Analysis, using Spearman's correlation test, was conducted for an association between the total number of muscle sarcocysts present and the hunter's assessment of body condition of the bird.

Results

Hunter questionnaire

Responses were received from a total of 948 individuals, with 820 and 128 replies being provided by the BASC email and Facebook links respectively.

Of these respondents, 136 (14.4%) had seen rice-grain lesions with 211 cases reported in 10 wildfowl species (Fig. 1A and B). The regions with the highest number of reports were Scottish Highlands and Islands (n=24), East Anglia (n=21) and Lancashire (n=20) (Fig. 2). The number of reported cases increased from the 2010/11 shooting season onwards (Fig. 3). Analyses of the data assessed the relationship between hunter harvest effort, hunting trends and observation of infection (Table 1). The average annual duck bag was significantly higher in those who had seen *Sarcocystis* infection (61.7 ducks per year), compared with those who hadn't (52.8 ducks per year).

	Hunting effort		
	Increased	**Remained constant**	**Decreased**
Yes, observed wildfowl *Sarcocystis* infection	26	53	24
Never observed wildfowl *Sarcocystis* infection	128	357	120

Table 1: Collated results comparing hunting effort and case observation using individual assessment of hunting trend (harvested numbers per year), over the 2009/10-2016/17 shooting seasons. No statistical association could be demonstrated (p=0.33), between hunting trend and the observation of cases, using Fisher's exact test.

Assessment of hunters' views on potential disease emergence, when contrasted with the years in which they began shooting, showed no association (sTable 2).

Online reporting

A total of 147 suspected cases of rice-breast disease in UK wildfowl were submitted between the 2015/16 and 2018/19 shooting seasons, with seven, 35, 47 and 58 birds being reported respectively per season. Seven species of wildfowl were reported, namely: mallard (41%), wigeon (25%), teal (24%), greylag goose (*Anser anser*) (3%), gadwall (3%), pintail (2%), and northern shoveler (2%). The Pearson's chi-square test suggested there was a significant difference between the observed number of quarry species shot and expected bag stats ($X^2_{(DF = 5)} = 536.01$, $p < 0.005$).

Assuming correct gender determination of shot birds, the majority of submissions were male (69%), with fewer females (27%) and a portion of birds of unknown sex (4%). Birds were primarily found to be in good body condition (67%), with some respondents reporting birds with fair (27%) and thin (6%) body condition. No respondents reported finding rice-grain lesions in emaciated birds.

The regions with the highest number of submissions were the Scottish Highlands & Islands (n=23), North East Scotland (n=18), North East England (n=18) and East Anglia (n=17), with these four regions comprising over 51% of the total submissions.

Accompanying comments from some of the submissions indicated that in many years/a lifetime of shooting these were the first cases they had seen, and some (n=6) reporting multiple cases in one day of shooting.

Molecular identification

Molecular analysis on the 14 hunter submitted samples resulted in 11 of the samples having PCR quality DNA and positive results for at least one of the three assays (Table 2, sTable 3), with varied sample quality resulting in mixed PCR results. Five host species were sampled, all of which presented at least one positive by PCR.

Species	Number submitted	Gender		PCR result	Body condition assessment	
		Male	Female	No. positive	Positive	Negative
Gadwall	1	1	0	1	Thin	N/A
Mallard	7	5	2	5	Good to fair	Good to thin
Pintail	1	1	0	1	Good	N/A
Teal	1	1	0	1	Fair	N/A
Wigeon	3	1	2	3	Good to fair	Good

Table 2: Summarised details of the submitted infected birds. Body condition assessment was undertaken by hunters in the field, using the descriptions defined in the supplementary material.

Eleven samples yielded 18S rRNA gene amplicons, including seven and 11 from the ERIB1/10 and SarAF/AR primer pairs, respectively (accession numbers LT992317-34). Three samples were negative by PCR for all assays, likely due to excessive degradation and/or prior storage in formalin. Using BLASTn all sequences were found to share the greatest similarity with sequences published for *S. rileyi*, including 99-100% identity across the full amplicon length with European *S. rileyi* isolates from Norway (11) and Lithuania (10) (GenBank accession numbers KJ396583.1 and HM185742.1). Just three of 14 samples yielded ITS-1 amplicons despite repeated testing (from two mallard, one wigeon; accession numbers LT992314-16), possibly limited by template DNA quality. All three sequences were identical. Using BLASTn the ITS-1 sequence was found to be identical to Norwegian isolate Sm 1.60 (KJ396584.1), with just two nucleotide substitutions when compared to a *S. rileyi* isolate obtained from a mallard in the USA (GU188427.1) (19).

Phylogenetic relationships

The ITS-1 sequences reported here were compared with a panel of published *Sarcocystis* sequences and *T. gondii*, with the latter used as an out-group (reference sequences as indicated in Fig. 4). The Tamura-3 model was found to be optimal. The resulting ML phylogenetic tree presented a topology comparable to versions created using NJ and MP; hence only one is shown. The tree showed a distinct clade including our UK *S. rileyi* isolates and previously published European (n=2) and North American (n=1) sequences. These *S. rileyi* isolates form a sister grouping to other *Sarcocystis* species which also parasitise wildfowl, *S. albifronsi* and *S. anasi*.

Pectoral muscle histopathology

Examination by light microscopy revealed myopathies in five of the 12 assessed pectoral samples (sTable 4), with myositis being the most frequent histological diagnosis. Inflammation local to a sarcocyst was seen in three of the infected birds, with two of these birds having moderate or severe inflammation scores. However, seven of the birds had no discernible muscle lesions associated with infection.

The inflammatory infiltrate, observed in three myositis cases, was composed of lymphocytes and plasma cells, with occasional macrophages and multinucleate giant cells being seen. All of the sarcocysts that had associated inflammatory responses were in a state of degeneration (Fig. 5A).

The most severe lesions were seen in a female wigeon that had a multifocal necro-granulomatous monophasic myositis associated both with local degenerate sarcocysts and within distant muscle fibre bundles (Fig. 5B and C). A mononuclear inflammatory infiltrate (as above) was seen invading a ruptured and/or degenerate sarcocyst, with the cyst wall losing its defining mural structure. Within the sarcocyst, there was loss of bradyzoite definition with abundant central necrosis associated with the inflammatory infiltrate. Surrounding the ruptured sarcocyst were extensive local areas of necrotic myofibres. Moderate to severe multifocal myositis was also observed distant to the sarcocysts with a mononuclear infiltrate surrounding myofibre bundles and causing multifocal atrophy and degeneration (Fig. 5C).

Myofibre atrophy and muscle fibrosis were less frequently observed lesions. Myofibre compression and subsequent mild atrophy was associated with the space-occupying effect of larger sarcocysts (Fig. 5D).

When statistically assessed, using Spearman's correlation test, no significant association was detected between reduced body condition and number of sarcocysts present histologically in a sample (S=298.56, p=0.892).

Discussion

The questionnaire and online reporting system components of this work provide a broad overview of UK wildfowl *S. rileyi* infection and represent the first UK based assessment. The engagement and utilisation of the general public in wildlife disease surveillance schemes allows for benefits to all involved stakeholder groups (20), accepting that the strategy brings some limitations and biases.

The total number of cases identified here is undoubtedly an under-representation of *S. rileyi* infection in UK shot wildfowl. UK hunters are not legally obliged to register centrally, join a shooting organisation nor report annual shooting bag statistics. Previous randomised BASC surveys indicate that 59.4% of members shoot wildfowl at least annually (21), offering a large group of surveyable stakeholders. Limitations, however, include historical recall which may result in misreporting and lesion identification relying on visual assessment of the affected carcasses having the pectoral muscles removed, or at least the skin reflected, before cooking, as cooking renders the sarcocysts invisible (4).

The three most commonly infected species from our questionnaire and online reporting are also those most commonly shot in the UK (22), with an estimated 880,000 mallards, 80,000 teal and 29,000 wigeon being taken annually at inland shoots (14) and lower numbers (~1,000 of each species) being harvested at Crown Estate foreshore shoots (23). However, the results suggest that the number of birds shot differs from the expected values as described by National Gamebag Census (14). This indicates there may be a possible underrepresentation of mallard and overrepresentation of wigeon, within our reported sample, which could be due to an increased risk of infection in this latter species. This dabbling duck is somewhat different from the others and is classified within a separate genus and has a different feeding ecology.

Responses to the questionnaire and online reporting system indicated that there is a male bias in the number of cases reported although this may reflect the shooting bag rather than susceptibility. Previous studies indicate wigeon shooting can result in biased sampling of adult males (24), and a male bias may also be seen with certain shooting techniques, such as when using decoys (25). Areas with an increased number of reported cases may additionally represent regions with increased hunting activity, suitable wildfowling wetlands and shooting permissions. The marked increasing number of cases, in particular reported from 2010/11 in the questionnaire survey may be associated with emergence of an infectious agent, or be potentially associated with increasing public awareness about the infection or an artefact of recall bias (26).

The amplicon sequencing confirmed the presence of *S. rileyi* in UK wildfowl. ~~Some confusion regarding the identity of *S. rileyi* does persist given that the morphological descriptions have been based on experimentally infected ducks and the North American restricted definitive host the striped skunk, whereas molecular sequences of European cases are based on sporocysts of naturally infected foxes and raccoon dogs (12).~~ Despite a limited sample size of submitted wildfowl, we have identified five dabbling duck species as potential UK intermediate hosts. Whilst macroscopic *Sarcocystis* infection has previously been documented in all of the submitted host species (15,27) and *S. rileyi* identified by molecular methods in three of the submitted host species (3,19), this work appears to be the first record of *S. rileyi* infecting gadwall, confirmed by molecular analysis.

Since the partial ITS-1 sequence reported here was identical or very similar to the previously published *S. rileyi* isolates from Lithuania, Norway and USA, it is difficult to determine whether these countries are the potential source of infection within the flyway of UK wildfowl or whether a lack of global genetic ITS-1 diversity exists (3). Although comprehensive European wildfowl sarcocystosis research is lacking (1), all of the submitted species are primarily winter migrants to the UK, with smaller numbers breeding but noting that mallard in particular may not be migratory, instead coming from UK stocks released for shooting purposes. The reporting of cases of *S. rileyi* in ducks from Lithuania, Norway and Finland in 2014 (3,11) may suggest this is a relatively recent infection within birds of the North West European flyway and/or there has been a change in transmission. The source of this parasite and the definitive mammalian hosts involved remains unknown, recognising common mammalian predators of wildfowl further up the flyway include foxes, American mink (*Neovison vison*) and raccoon dogs. Further research is required including into the potential roles of the increasing European populations of raccoon dogs and raccoons (*Procoyn lotor*), invasive alien species which have been shown experimentally to be definitive hosts of *S. rileyi* (12,28).

Two cases of granulomatous myositis associated with wildfowl *Sarcocystis* infection have been described previously (8), however the sarcocysts were not typed nor was cyst histopathology described. *Sarcocystis*

associated myositis has been seen to cause weakness and lethargy in domestic fowl (29), it remains unclear as to the host health impacts of *S. rileyi* and further research is required. Despite 5/12 birds having varying myopathies, the remaining birds had no microscopic inflammation surrounding the cysts, however some variation in slide quality may occur between samples, as a result of autolysis or freeze-thaw damage, making histological interpretation less standardised. It is notable that reported cases were generally birds in good health as determined by condition score.

This work highlights an emerging issue to the hunting and conservation communities and provides much opportunity for further research into *S. rileyi* infection in free-living UK and European wildfowl species. The implications of infection and described myopathy upon host health, fitness and survival of these migratory waterbirds are key points for consideration in future study.

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