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High prevalence of *Sarcocystis calchasi* sporocysts in European *Accipiter* hawks

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ABSTRACT

The emerging *Sarcocystis calchasi* induces a severe and lethal central nervous disease in its intermediate host, the domestic pigeon (*Columba livia* f. *domestica*). Experimental studies have identified the Northern goshawk (*Accipiter g. gentilis*) as final host. Phylogenetically closely related European sparrowhawks (*Accipiter n. nisus*) and wood pigeons (*Columba palumbus*) have been found to harbor genetically closely related *Sarcocystis* spp. However, data on the prevalence and potential interspecies occurrence of these parasites are lacking. Here, we report that European *Accipiter* hawks (Accipitrinae) are highly infected with *S. calchasi*, *S. columbae* and *Sarcocystis* sp. *ex A. nisus* in their small intestine. Thirty-one of 50 (62%) Northern goshawks necropsied during 1997–2008 were positive for *S. calchasi* in a newly established species-specific semi-nested PCR assay based on the first internal transcribed spacer region. Unexpectedly, 14 of 20 (71.4%) European sparrowhawks tested also positive. In addition, birds of both species were found to be infested with *S. columbae* and an, as yet, unnamed *Sarcocystis* sp. recently isolated from European sparrowhawks. These findings raise new questions about the host specificity of *S. calchasi* and its high virulence in domestic pigeons, since sparrowhawks only rarely prey on pigeons. Notably, isolated sporocysts from both infected *Accipiter* spp. measured 8 μm × 11.9 μm, precluding a preliminary identification of *S. calchasi* in feces of *Accipiter* hawks based on morphology alone. Importantly, three of four Northern goshawks used in falconry tested positive for *S. calchasi*. In conclusion, the results indicate that both European *Accipter* spp. in Germany serve as natural final hosts of *S. calchasi* and suggest that falconry and pigeon sport may serve as risk factors for the spread of this pathogen in domestic pigeons.

1. Introduction

Sarcocystis calchasi is an emerging protozoan parasite that can induce a fatal central nervous disease in domestic pigeons (*Columba livia* f. *domestica*; Olias et al., 2009). Experimental studies have identified the Northern goshawk (*Accipiter g. gentilis*) as final host (Olias et al., 2010b). The clinical course of the natural disease and the post mortem lesions were reproduced experimentally (Olias et al., 2009, 2010b). Notably, central nervous signs of the pigeons were very similar to those seen after infections with the highly pathogenic avian influenza (HPAI) virus H5N1, pigeon paramyxovirus type 1 (PPMV1) and *Salmonella* Typhimurium var. Copenhagen (Faddoul and Fellows, 1965; Alexander et al., 1984a; Alexander et al., 1984b; Klopfleisch et al., 2006).

Recent phylogenetic analyses (Olias et al., 2010b) give rise to the assumption that the parasite might be present elsewhere in the Northern Hemisphere (Holarctic) where the different subspecies of the Northern goshawk and the domestic pigeon share their distribution area (del Hoyo

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et al., 1997; Ferguson-Lees et al., 2001). However, data on the natural prevalence of the parasite in its final host are lacking. Recently, the North American Cooper's hawk (*Accipiter cooperi*) has been assumed to potentially harbor *S. calchasi* due to amplification of an identical 18S rRNA gene segment from the intestine of these raptors (Yabsley et al., 2009; Olias et al., 2010b) and raised questions about the host specificity of the parasite. *Accipiter* hawks of the Holarctic belong to the same genus within the subfamily Accipitrinae (Wink and Sauer-Gürth, 2004; Lerner and Mindell, 2005). Recent studies have identified two *Sarcocystis* spp. genetically closely related to *S. calchasi* from phylogenetically closely related intermediate and final hosts in Europe, the wood pigeon (*Columba palumbus*) and the European sparrowhawk (*Accipiter n. nisus*; Olias et al., 2010c). Birds of prey have been shown to act as final hosts for numerous *Sarcocystis* spp. (Lindsay and Blagburn, 1999; Svobodova et al., 2004; Yabsley et al., 2009). In particular, Northern goshawks harbor at least five putatively different *Sarcocystis* spp. (Holling and Fowle, 1955; Gottschalk, 1972; Cerna and Kvasnovska, 1986; Kolarova, 1986; Svobodova, 1996; Olias et al., 2010a). However, data on the life cycles of bird-infecting *Sarcocystis* spp. in general are scarce. Both, the goshawk and the sparrowhawk are the only two *Accipiter* spp. widely present throughout Europe and being the most common avian predators in the forested regions of the Palearctic (Ferguson-Lees et al., 2001). In contrast to the larger goshawk, the sparrowhawk with a male body mass averaging 144 g and females 264 g only infrequently hunts pigeons and doves, but feeds mainly on tits, trushes, finches, buntings and sparrows (Opdam, 1979; Newton, 1986).

Initial sequence analyses of the 18S and 28S rRNA genes among bird-infecting *Sarcocystis* spp. revealed highly conserved sequences with a pairwise distance (*p*-distance) of only 0.03–0.04 in the sequence of the variable D2 loop region of the 28S rRNA between *S. calchasi*, *S. columbae* and the, as yet, unnamed *Sarcocystis* sp. *ex Accipiter nisus*. Analysis of the highly variable internal transcribed spacer (ITS) region 1 in turn measured a *p*-distance of 0.165–0.195 (Olias et al., 2010c), making this region a promising target for species-specific PCR primers. The ITS-1 region has already been proven to be suitable to discriminate between phylogenetically closely related bird-infecting *Sarcocystis neurona* and *Sarcocystis falcatula* (Marsh et al., 1999; Mansfield et al., 2008) which show a high *p*-distance of 0.485 to 0.499 when compared with *S. calchasi* (Olias et al., 2009). The aim of the present study was therefore to evaluate fresh and archived samples of European *Accipiters* spp. by a species-specific PCR method for natural infection with *S. calchasi*, *S. columbae* and *Sarcocystis* sp. *ex A. nisus*. Here, we provide evidence that both European *Accipiter* hawks collectively act as natural final hosts for all three *Sarcocystis* spp.

2. Material and methods

2.1. Sources of Northern goshawks and European sparrowhawks

Forty-six free-ranging and four captive Northern goshawks (*Accipiter g. gentilis*) used in falconry were necropsied between 1997 and 2008. The birds were 24/50 (48%) female and 26/50 (52%) male. Twenty free-ranging European sparrowhawks (*Accipiter n. nisus*) were necropsied between 1997 and 2009. The birds were 11/20 (55%) female and 9/20 (45%) male. All birds originated from Northern Germany. In most cases the cause of death was trauma.

2.2. Light microscopy of Sarcocystis species

Tissue samples from the small intestine of all goshawks and sparrowhawks were fixed in 4% phosphate-buffered formalin, routinely embedded in paraffin and sections of 4–6 μm thickness were H & E stained. For morphologic characterization, sporocysts derived from intestinal scrapings of a subset of 23 goshawks and four sparrowhawks were purified as described elsewhere (Rommel et al., 1995).

2.3. DNA extraction and integrity

DNA was isolated from intestines, formalin fixed and paraffin embedded (FFPE) tissue blocks or purified sporocysts, respectively. For FFPE tissue, 15 slices of 4 μm thickness were deparaffinized for 10 min in 500 μl xylene and washed for 10 min in 500 μl 100% ethanol. After each step the mixture was centrifuged briefly and the solvent was removed. For sporocysts, a 1 ml aliquot of each purified sporocyst suspension was centrifuged for 10 min at 11,000 × *g*. DNA was extracted from the tissue and sporocyst pellets using overnight proteinase K digestion at 56 °C and affinity column separation following the instructions of the supplier (NucleoSpin Tissue, Macherey-Nagel, Düren, Germany). To evaluate the integrity of the isolated DNA of purified sporocysts and FFPE tissue samples, sequences encoding the cytochrome B gene and a part of the 28S rRNA gene (primers KL6a and KL5b) were amplified by PCR as described previously (Mugridge et al., 1999; Kocher et al., 1989). Genomic DNA derived from *Frenkelia microti* and from an uninfected, non-FFPE tissue sample of a Northern goshawk was included as a positive control for PCR amplification of the partial 28 rRNA gene and the cytochrome B gene, respectively.

2.4. Species-specific semi-nested PCR amplifications

Specific primer sites were identified by comparison of the ITS-1 regions of *S. calchasi*, *S. columbae* and *Sarcocystis* sp. *ex A. nisus* (GenBank accession no. FJ232948; GU253885; GU253886; Olias et al., 2009). PCR primers were designed using the GeneFisher 2.0 software (Table 1; Fig. 1; Giegerich et al., 1996). PCRs were conducted semi-nested for each *Sarcocystis* species. Primer pairs SCa1/SCa2, SCo1/SCo2 and SNi1/SNi2, respectively, were used for initial amplification. Amplicons were diluted 1:500 for subsequent amplification with primer pairs SCa1/SNCa3, SCo1/SNCo3 and SNi1/SNNi3, respectively. Predicted amplicon sizes were 136 bp for *S. calchasi*, 129 bp for *S. columbae* and 124 bp for *Sarcocystis* sp. *ex A. nisus*. All amplification reactions were performed in 50 μl reaction mixtures containing 200 pM of each primer, 10 μl of 5 × Green Flexi Buffer, reaction buffer, dNTP mix (0.2 mM each), 1.5 mM $MgCl_2$ and 1.25