The red fox (*Vulpes vulpes*) and the arctic fox (*Vulpes lagopus*) are definitive hosts of *Sarcocystis alces* and *Sarcocystis hjorti* from moose (*Alces alces*)

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SUMMARY

The aim of this study was to determine whether foxes might act as definitive hosts of *Sarcocystis alces* in moose. In 2 experiments, 6 silver foxes (*Vulpes vulpes*) and 6 blue foxes (*Vulpes lagopus*) were fed muscle tissue from moose containing numerous sarcocysts of *S. alces*, and euthanased 7–28 days post-infection (p.i.). Intestinal mucosal scrapings and faecal samples were screened microscopically for *Sarcocystis* oocysts/sporocysts, which were identified to species by means of species-specific primers and sequence analysis targeting the ssu rRNA gene. All foxes in both experiments became infected with *Sarcocystis*; the oocysts were fully sporulated by 14 days p.i., containing sporocysts measuring 14–15 × 10 μm. Molecular identification revealed that the oocysts/sporocysts belonged to 2 species, *S. alces* and *Sarcocystis hjorti*, although sarcocysts of *S. hjorti* were only identified in moose subsequent to the infection of foxes. In the first experiment, all 8 foxes also became infected with a *Hammondia* sp. derived from moose, shedding unsporulated, subspherical oocysts, measuring 10–12 μm in diameter, from 6–7 days p.i. onwards. The study proved that canids (the red fox and arctic fox) are definitive hosts for *S. alces* and *S. hjorti*, as had been inferred from the phylogenetic position of these species.

Key words: *Hammondia*, *Sarcocystis alces*, *Sarcocystis hjorti*, *Vulpes lagopus*, *Vulpes vulpes*, ssu rRNA gene.

INTRODUCTION

Sarcocystis species have an obligatory 2-host life cycle with carnivores as definitive hosts and mostly omnivores or herbivores as intermediate hosts (Dubey *et al*. 1989). A single herbivore may serve as the intermediate host for several *Sarcocystis* species, often concurrently, and this is very common in cervids. Thus, 5 *Sarcocystis* species have been described from moose (*Alces alces*) in Norway; 3 species (*Sarcocystis alces*, *Sarcocystis ovalis*, and *Sarcocystis scandinavica*), on the basis of both cyst morphology and molecular data, and 2 species (*Sarcocystis* sp. Type E and Type D) only on the basis of unique DNA sequences (Dahlgren and Gjerde, 2008). However, a species with the same ssu rRNA gene sequence as the unnamed *Sarcocystis* sp. Type E in moose (Dahlgren and Gjerde, 2008) was later described by both cyst morphology and molecular data from red deer and named *Sarcocystis hjorti* (Dahlgren and Gjerde, 2010). It then also became obvious that sarcocysts of *S. hjorti*, as seen in red deer, were morphologically indistinguishable from those of *Sarcocystis alceslatrans*, which had been obtained from 2 Canadian moose and examined by light microscopy by Dahlgren and Gjerde (2008). Molecular analysis did, however, prove that *S. alceslatrans* was different from *Sarcocystis* sp. Type E. *S. alceslatrans* had originally been reported from moose in USA (Dubey, 1980) and Canada (Colwell and Mahrt, 1983), and had been found to use coyotes and dogs as definitive hosts (Dubey, 1980; Colwell and Mahrt, 1983). By contrast, the life cycle of all *Sarcocystis* species identified in Norwegian moose was unknown.

S. alces was found to be the most prevalent *Sarcocystis* species in Norwegian moose (Dahlgren and Gjerde, 2008). In addition, several moose in that study were heavily infected, which is likely to have had a negative impact on the health of the animals. The high prevalence and infection intensity of *S. alces* suggested that the definitive hosts were common in Norwegian forests. Moreover, the phylogenetic position of *S. alces* indicated that canids were its most likely definitive hosts (Dahlgren and Gjerde, 2008). Since red foxes (*Vulpes vulpes*) commonly feed on carcasses of moose that have died from accidents or disease, we were particularly interested in using this species in the transmission experiments, but we also included the arctic fox (*Vulpes lagopus*) in the study. Thus, the aim of this study was to determine whether the red fox and the arctic fox would act as definitive hosts for *S. alces*, through the identification of any sporocysts/oocysts in the intestine or faeces of the

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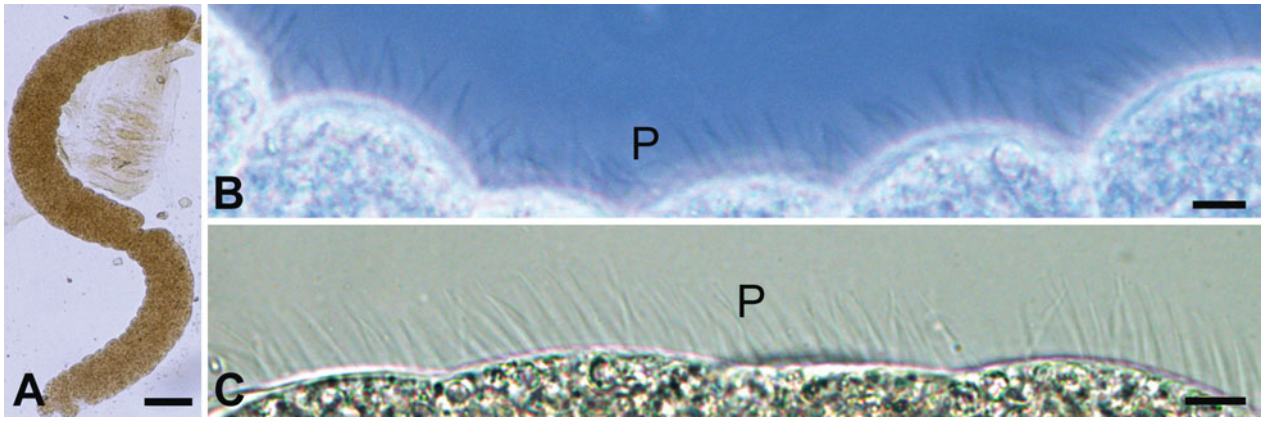

Fig. 1. *Sarcocystis hjorti* in moose. (A) Nearly complete cyst, which was subsequently determined by sequence analysis to belong to *S. hjorti*. (B) Detail from surface of cyst in (A), showing delicate hair-like protrusions. Phase contrast. (C) Another small cyst with hair-like protrusions consistent with *S. hjorti*. Scale bars = 150 μm in (A); 10 μm in (B) and (C).

inoculated foxes by molecular methods, using *Sarcocystis* sequence data obtained in previous studies for this purpose (Dahlgren and Gjerde, 2007, 2008, 2009, 2010).

MATERIALS AND METHODS

Experimental animals

A total of 12 foxes were used in 2 separate experiments; 8 foxes in Experiment 1 (October 2007), and 4 foxes in Exp. 2 (October 2008). An equal number of silver foxes (colour mutants of the red fox (*Vulpes vulpes*)) and blue foxes (colour mutants of the arctic fox (*Vulpes lagopus*)), were used in each experiment. In Exp. 1, all foxes were females, 1–5 years old, whereas in Exp. 2, 2 female silver foxes, 1–2 years old, and 2 male blue foxes, about 6 months old, were used. All foxes had been born and reared at the Unit for Fur-bearing Animals at the Animal Production Experimental Centre at the Norwegian University of Life Sciences, Ås, Norway. The foxes were caged individually and fed their ordinary food throughout the experimental period, except on day 0, when they were only fed fresh muscle tissue. The ordinary food consisted mainly of heat-treated and ground offal, mostly from broilers. The foxes had never eaten fresh muscle tissues before the experiments. The foxes were killed with electrocution according to standard euthanasia methods. Both experiments had been approved by the Norwegian Animal Research Authority (NARA).

Collection and preparation of infective material

Experiment 1. Fresh portions from the oesophagus, diaphragm, and abdominal muscles were obtained from 5 moose that had been killed in Nordmarka, near Oslo, during the regular hunt in October 2007. The carcasses were processed at an abattoir-like plant and had been kept there at around 4 °C, for a maximum of 7 days before sampling. Muscle tissues from moose were screened for sarcocysts under a stereomicroscope. A presumptive species identification of several cysts of each type, as seen *in situ*, was made by excising the cysts and examining their surface morphology in wet mounts under a light microscope. A few sarcocysts of each cyst type were also excised and their species identity subsequently determined by molecular methods as described previously (Dahlgren and Gjerde, 2007). Samples from all animals contained cysts (from a few to numerous) consistent with those of *S. alces*; samples from 3 animals contained a few cysts of *S. ovalis* and, in 1 animal, a single cyst with hair-like protrusions was found (Fig. 1). The muscle tissues from all moose were cut into small pieces, thoroughly mixed and divided into 8 portions of about 200 g each, which were fed to each of 8 foxes the next day in a single feeding.

Experiment 2. Fresh portions from the oesophagus, diaphragm, and abdominal muscles were obtained from 7 moose that had been killed during the regular hunt in Nordmarka, near Oslo, in October 2008. Samples were collected at the same processing plant as in 2007, from 1 moose that had been killed just a few hours earlier, from 2 carcasses that had been kept at about 4 °C for a maximum of 4 days, and from offal from 3–4 carcasses, which had been kept in a container outside the plant for 3–4 days at about 10 °C. The muscle samples were examined as in Exp. 1. In the most recently killed moose, numerous cysts of *S. alces* and a few cysts of *S. ovalis* were seen. In the other samples, only a few cysts of these two species were observed. The muscle tissues were cut into small pieces, then thoroughly mixed and split into 4 portions of about 700 g each, which were fed to each of 4 foxes the next day in a single feeding.

Animal monitoring and blood sampling

The animals were monitored every day for clinical symptoms, faecal consistency and appetite. Blood

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samples (serum and full blood) were taken from *Vena cephalica* from all experimental animals, starting at day 0 (control samples), and collected with 7 days interval until euthanasia. The blood samples were analysed at the Central Laboratory at the Norwegian School of Veterinary Science. Serum samples were examined for total protein, albumin, globulin, creatinine, bile acids, total bilirubin, cholesterol, glucose, urea, enzymes (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatinine kinase, amylase, lipase), minerals (inorganic phosphate, calcium, sodium, potassium, chloride), and hormones (cortisol). Full-blood samples were examined for concentration of erythrocytes and different types of leucocytes (lymphocytes, neutrophils, monocytes, eosinophils, basophils). Blood parameters of an uninfected fox sampled concurrently with the experimental foxes, control samples of all experimental animals on day 0, and reference values from cats and dogs were used as estimates of normal range values for the foxes.

Faecal examination

In 2007, faecal sampling started on day 6 after feeding and faeces were collected every day until euthanasia. In 2008, due to a misunderstanding by the staff at the research facility, daily samples were only collected on days 6 and 7, whereas faeces from days 8–15 were combined into 1 pooled sample per animal. The faecal samples were stored at 4 °C for 1–3 months and then examined under a light microscope after flotation of the samples in saturated sucrose. Small portions (about 0·5 ml) of each faecal sample were transferred to 1·5 ml Eppendorf tubes and stored at −20 °C for subsequent molecular analysis.

Intestinal examination

Two randomly selected animals, 1 silver fox and 1 blue fox, were killed on days 7, 14, 21, and 28 post-inoculation (p.i.) in Exp. 1, and on days 8 and 15 p.i. in Exp. 2. The entire intestine was removed, cut open lengthwise, and the contents and mucosal lining examined for parasites along the entire length of the intestine (about 180–200 cm). Samples from the intestinal mucosa were taken at approximately 10 cm intervals, by scraping with a scalpel blade. The scrapings were smeared onto a microscope slide and examined for *Sarcocystis* oocysts/sporocysts under a light microscope. Pictures were taken with a Leica MPS60 photomicrography system (Exp. 1) or a Leica DC480 digital camera (Exp. 2).

In Exp. 2, a few oocyst-positive scrapings were directly transferred from the microscope slide to 1·5 ml Eppendorf tubes containing about 0·5 ml of distilled water and stored at −20 °C until DNA isolation and molecular examination. The remaining intestinal mucosa from the foxes in Exp. 2, and all mucosal samples in Exp. 1, were treated as follows: areas where oocysts had been observed microscopically were scraped with a scalpel blade and the mucosal lining was transferred to 50 ml plastic tubes containing either 70% alcohol or distilled water. The samples were stored at −20 °C until molecular examination.

DNA isolation

Two samples from each fox were examined by molecular methods. DNA was isolated from faecal and/or mucosal samples containing oocysts/sporocysts.

Intestinal mucosa containing *Sarcocystis* oocysts, which had been stored frozen in distilled water, was thawed and portions of about 0·7 ml of this mixture were transferred from the 50 ml tubes to 1·5 or 2·0 ml Eppendorf tubes. These tubes were centrifuged at 12 000 ***g*** for 1 min, and the supernatant was discarded. A few samples that had been stored in 70% alcohol were transferred to 1·5 ml Eppendorf tubes, and left open at room temperature until most of the fluid had evaporated. To evaluate whether temperature treatment would improve DNA recovery, a few samples were incubated in TE-buffer at 100 °C for 60 min. Genomic DNA from the samples was isolated either by using QIAamp® DNA Mini Kit (Qiagen GmbH, Germany) according to the manufacturer's tissue protocol, or by using QIAamp® Stool DNA Mini Kit (Qiagen GmbH, Germany) according to the protocol described for the extraction of DNA from *Tritrichomonas foetus* (Gookin *et al.* 2002). The 'Stool kit' and associated protocol were also used for DNA isolation from faecal samples that had been stored frozen in Eppendorf tubes. The samples containing intestinal scrapings that had been transferred directly to, and stored at −20 °C in 1·5 ml Eppendorf tubes, were thawed, centrifuged for 60 s at 12 000 ***g***, and the supernatant decanted. DNA was then isolated using QIAamp® DNA Mini Kit, according to the manufacturer's tissue protocol.

DNA amplification and sequencing

The ssu rRNA gene was selected as a target molecule to test all DNA samples for the presence of coccidian DNA by PCR using forward primer ERIB1 (Barta *et al.* 1997) and reverse primer S4r (Fischer and Odening, 1998). PCR products were separated on 1% agarose gels and visualized under UV light after staining with ethidium bromide to check for appropriately sized products. To verify the results, positive PCR products of 1 DNA sample from each fox were sent for sequencing to Eurofins MWG Operon, Germany. Additional primers used in the sequencing reactions were S3f, S5f (Fischer and Odening, 1998) and Primer B (Fenger *et al.* 1995). PCR protocols

Table 1. Specific primers developed to selectively amplify regions of the ssu rRNA gene of *Sarcocystis alces* and *Sarcocystis ovalis*/*S. hardangeri*

Primer name*	Primer sequence (5′→3′)	Species specificity of primer
SD1f	TGCGGAAATATCCTTTTTCG	*S. alces*
SD1r	GCATATATTGGACTTGCGCC	*S. alces*
SD2f	TGTATTGGACTACCGTGGCA	*S. alces*
SD3f	TATACGGCGAAACTGCGAAT	*S. ovalis* and *S. hardangeri*
SD3r	GACCATGTGCACACCCATTA	*S. ovalis* and *S. hardangeri*

* f = forward; r = reverse.

and DNA purification have been described previously (Dahlgren and Gjerde, 2007). Vector NTI Advance 11 software (Invitrogen, Scotland) was used to assemble sequence information from forward and reverse primers into contiguous sequences.

All DNA samples were also tested for the presence of *Hammondia* DNA, using the *Hammondia* specific primers AT264/AT9, which target the alpha tubulin gene (Abel *et al*. 2006), followed by examination for PCR products on agarose gels. The identity of the coccidia present in a few samples could not be clearly resolved by ssu rRNA gene sequencing alone, and these samples were therefore checked for the presence of *Neospora caninum*-DNA, using the *N. caninum* specific primers Np21/Np6 (Yamage *et al*. 1996), which amplify part of the pNc5-gene. DNA from *N. caninum*, *S. alces, S. hjorti*, and the *Hammondia* sp. detected in this study were included in the PCR as positive and negative controls.

Design of species-specific ssu rRNA gene primers for S. alces *and* S. ovalis

Species-specific primers were designed for those *Sarcocystis* species which were readily seen in the moose musculature, i.e., *S. alces* and *S. ovalis*. Primer-BLAST was used to search for suitable primers along the entire ssu rRNA gene with the following parameters for primer design: optimal melting temperature of 60 ± 3 °C, optimal primer length of 20 bases, PCR product of minimum 200 bases and maximum 1000 bases. Primer specificity was checked using the 'nr' Nucleotide Sequence Database at NCBI. Three sense and 2 antisense oligonucleotides were designed and obtained from Invitrogen™ (Table 1). The suitability of the primers was tested by using for each reaction: 3 μl DNA template, 12·5 μl HotStarTaq Master Mix (Qiagen GmbH, Germany), 10 pmol of each primer, 4 μg bovine serum albumin, and RNase-free water to make a final volume of 25 μl. The annealing temperature was optimized by increasing the temperature stepwise from 52 °C to 60 °C. Sensitivity of the PCR assay was determined by diluting genomic DNA preparations, starting with a 1 : 1 dilution and increasing the proportion of water stepwise to a final 1 : 100 dilution. PCR products (10 μl) were separated and visualized on 1% agarose gels. In addition to the specificity check by Primer-BLAST, the primer pairs were also tested with DNA from all *Sarcocystis* species that we previously had characterized or identified by molecular methods from cervids (*S. alces, S. alceslatrans, S. hjorti, Sarcocystis gracilis, Sarcocystis grueneri, Sarcocystis hardangeri, S. ovalis, Sarcocystis oviformis, Sarcocystis rangi, Sarcocystis rangiferi, S. scandinavica, Sarcocystis tarandi, Sarcocystis tarandivulpes*, and *Sarcocystis* sp. Type D of moose) (Dahlgren and Gjerde, 2007, 2008, 2009, 2010). All primers were also tested using DNA from the *Hammondia* sp. detected in this study and with fox-DNA obtained from a liver sample.

The faecal and mucosal samples from the foxes were finally tested using the following optimized PCR protocol. Each reaction mixture contained: 4 μl DNA template, 25 μl HotStarTaq Master Mix (Qiagen GmbH, Germany), 20 pmol of each primer, 8 μg bovine serum albumin, and RNase free water to make a final volume of 50 μl. PCR-cycling conditions were: initial hot start at 95 °C for 15 min, followed by 35 cycles of 94 °C for 40 s, 58 °C for 45 s, and 72 °C for 60 s; and a final extension at 72 °C for 10 min.

Examination of moose for sarcocysts of S. hjorti

Due to the finding of *S. hjorti*-infection in several foxes (see later), the oesophagus and/or portion of the diaphragm from an additional 11 moose were examined in October 2009, in an attempt to determine whether *S. hjorti* was a common species in moose and whether the cysts of this species could be differentiated from those of *S. alces in situ* at low magnification. The samples were obtained at the abattoir-like plant described above from moose killed in Nordmarka during the regular hunt in 2009. The tissue samples were examined under a stereomicroscope and a large number of sarcocysts were excised and further examined under a light microscope for hair-like surface protrusions consistent with those of *S. hjorti* in red deer (Dahlgren and Gjerde, 2010).

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RESULTS

Sarcocystis *species identified by light microscopy and molecular methods in muscle tissues from moose used in the experiments*

Experiments 1 and 2. Numerous 2–5 mm long and spindle-shaped cysts were found in the oesophagus, diaphragm, and abdominal muscles. The size, shape, and surface morphology of these cysts were consistent with those of *S. alces* (Dahlgren and Gjerde, 2008), and molecular examination of one such cyst, confirmed its identity as *S. alces* (100% identity with another *S. alces* sequence in GenBank, Accession number EU282018). A small cyst, measuring 3·0 × 0·15 mm, that was found in the muscles of one of the moose used in Exp. 1, had similar delicate hair-like protrusions, about 10 μm long (Fig. 1A, B) as cysts of *S. alceslatrans* of moose and *S. hjorti* of red deer (Dahlgren and Gjerde, 2008, 2010). Molecular examination revealed that this cyst belonged to the previously reported *Sarcocystis* sp. Type E in moose (Dahlgren and Gjerde, 2008), which more recently has been described as *S. hjorti* in red deer (Dahlgren and Gjerde, 2010) (100% identity with another *S. hjorti* sequence and *Sarcocystis* Type E sequence in GenBank, Accession numbers GQ250990 and EU282017). A few 1–2 mm long and oval cysts, consistent with those of *S. ovalis* in moose (Dahlgren and Gjerde, 2008) were also observed in the moose tissues used in both experiments.

Clinical observations

All foxes were clinically normal at the beginning of the experiments. Six of the 8 foxes in Exp. 1 had reduced feed intake, soft faeces and/or vomited between day 6 and 10 p.i. One fox in Exp. 2 had reduced feed intake and soft faeces on day 8 p.i., and 1 fox had diarrhoea and vomited on day 13 p.i. The blood parameters in each fox varied slightly between sampling days, but fluctuated within the same range as the control samples from foxes and also within the normal range for cats and dogs. No increase or decrease in any blood value seemed to be associated with the experimental infections.

Microscopic findings in faecal samples from foxes

Experiment 1. A few *Sarcocystis* sporocysts, measuring approximately 12·5 × 8 μm, were detected in the faeces of 1 silver fox on day 13 p.i. and in the faeces of another silver fox on day 14 p.i.; all other samples were negative for sporocysts (Table 2). Unsporulated *Hammondia* oocysts, about 10–12 μm in diameter (Fig. 2), were detected in the faeces of all foxes from days 6–8 p.i. onwards, except in 1 of the foxes killed on day 7 p.i., which, however, had numerous oocysts in the ileum contents at that time. The *Hammondia* oocysts were shed in large numbers during the first 1–5 days of patency, later on only in low numbers or intermittently, until the animals were euthanized (Table 2).

Experiment 2. No *Sarcocystis* oocysts/sporocysts were detected in any of the daily or pooled faecal samples, or in faeces retrieved from the colon of the 2 foxes killed on day 15 p.i. However, a single sporulated *Sarcocystis* oocyst was found by flotation of ileum contents from 1 of the foxes killed on day 15 p.i., indicating that oocyst shedding was about to begin (Table 2). A few unsporulated *Hammondia*-like oocysts were seen in the faeces of 3 of the foxes on day 8 p.i. In 1 of these foxes, a few large, oval oocysts, measuring about 35 × 27 μm, and resembling those of *Isospora canivelocis*, were also found on day 8 p.i.

In both experiments, a few ascarid-type nematode eggs and oocysts without a recognizable sporont inside were seen in some samples. These were considered to be spurious parasites that the foxes had ingested with their ordinary feed, which contained viscera of slaughtered animals.

Microscopic findings in intestinal scrapings from foxes at necropsy

Experiment 1. A few scattered unsporulated, ellipsoidal *Sarcocystis* oocysts, measuring about 17·5 × 12·5 μm (Fig. 3A), were seen in the anterior part of the intestine in both foxes killed on day 7 p.i. (Table 2). A moderate number of sporulated, thin-walled *Sarcocystis* oocysts were seen in the mucosa throughout the length of the small intestine of all animals killed on day 14, 21, and 28 p.i. (Fig. 3C). The 2 sporocysts inside the oocysts each measured around 14–15 × 10 μm. Each sporocyst contained 4 sporozoites and a compact granular residual body.

Numerous unsporulated, subspherical *Hammondia* oocysts were observed in the mucosa along the entire posterior half of the small intestine in both foxes killed on day 7 p.i. A few *Hammondia* oocysts were also found in the ileum of 1 fox killed on day 14 p.i. (Table 2).

Experiment 2. A moderate number of unsporulated and partly sporulated, ellipsoidal *Sarcocystis* oocysts (Fig. 3B), measuring 15–20 × 10–15 μm, were seen along almost the entire length of the small intestine in both foxes killed on day 8 p.i. (Table 2). A moderate number of sporulated *Sarcocystis* oocysts, measuring 15–16 × 20 μm, were seen in the small intestinal mucosa of both animals killed on day 15 p.i. (Fig. 3D). The 2 sporocysts inside the oocysts each measured approximately 15–16 × 10 μm and had the same general morphology as in Exp. 1.

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Table 2. Experimental infections of foxes with *Sarcocystis*-infected muscle tissue from moose (Microscopic and molecular findings in faecal samples and mucosal scrapings from the small intestine.)

Fox	Euthanised on day[1]	Microscopic findings in faeces on various days after infection	Microscopic findings in the small intestinal mucosa at necropsy	Molecular findings in samples from faeces (F) and intestinal mucosa (M)
Blue fox[2] 1-07	7	*Hammondia* oocysts days 6 and 7 (++ [3] 7).	A few scattered unsporulated *Sarcocystis* oocysts throughout the anterior 25 cm. Numerous unsporulated *Hammondia* oocysts in the posterior part.	*S. alces* (M), *Hammondia* (M)
Silver fox 1-07	7	*Hammondia* oocysts day 7 (++ 7).	A few unsporulated *Sarcocystis* oocysts, mainly in the anterior 25 cm. Numerous unsporulated *Hammondia* oocysts in the posterior part.	*S. alces* (M), *S. hjorti* (M), *Hammondia* (M)
Blue fox 2-07	14	*Hammondia* oocysts days 6–13 (++ 7, 8).	A few sporulated *Sarcocystis* oocysts throughout the anterior 125 cm.	*Hammondia* (M)
Silver fox 2-07	14	*Hammondia* oocysts days 8–14 (++ 8). *Sarcocystis* sporocysts day 13.	Numerous sporulated *Sarcocystis* oocysts along the anterior 125 cm. A few unsporulated *Hammondia* oocysts in the posterior portion.	*S. alces* (M), *Hammondia* (M)
Blue fox 3-07	21	*Hammondia* oocysts days 7–20 (++ 7–11).	A few scattered sporulated *Sarcocystis* oocysts throughout the anterior 150 cm.	*Hammondia* (M)
Silver fox 3-07	21	*Hammondia* oocysts days 7–17 (++ 7, 8). *Sarcocystis* sporocysts day 14.	A few sporulated *Sarcocystis* oocysts throughout the anterior 125 cm.	*S. hjorti* (M + F), *Hammondia* (M + F)
Blue fox 4-07	28	*Hammondia* oocysts days 6–28 (++ 7).	A few sporulated *Sarcocystis* oocysts throughout the small intestine.	*Hammondia* (M)
Silver fox 4-07	28	*Hammondia* oocysts days 7–28 (++ 8–11).	A few scattered sporulated *Sarcocystis* oocysts throughout the anterior 175 cm.	Negative (M)
Blue fox 1-08	8	A few *Hammondia*-like oocysts.	A few unsporulated/partly sporulated *Sarcocystis* oocysts throughout the small intestine.	*S. alces* (M), *S. hjorti* (M)
Silver fox 1-08	8	No oocysts or sporocysts.	A few unsporulated/partly sporulated *Sarcocystis* oocysts throughout the small intestine.	*S. hjorti* (M) *Isospora* or *Toxoplasma* DNA[4] (M)
Blue fox 2-08	15	A few *Hammondia*-like oocysts day 8.	Sporulated *Sarcocystis* oocysts throughout most of the small intestine.	*S. hjorti* (M)
Silver fox 2-08	15	A few *Hammondia*-like oocysts day 8.	Sporulated *Sarcocystis* oocysts throughout the small intestine.	*S. hjorti* (M)

[1] Day refers to day after feeding moose tissues.
[2] Blue fox = arctic fox, silver fox = red fox.
[3] ++ Days with high numbers of *Hammondia* oocysts.
[4] Probably derived from the food, which included offal.

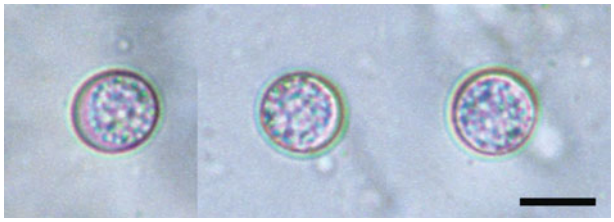

Fig. 2. Unsporulated oocysts of *Hammondia* sp. from small intestinal contents of a blue (arctic) fox 7 days post-feeding with muscle tissue from moose. Scale bar = 10 μm.

Identification of parasite stages in faeces or intestinal scrapings by molecular methods

DNA recovery from faeces or intestinal scrapings. DNA extraction from *Sarcocystis* oocysts/sporocysts in both the intestinal mucosa and faeces was most efficient with the QIAamp® DNA Mini Kit, using the standard 'tissue protocol' as described by the manufacturer. DNA isolation from faeces or from the intestinal mucosa using QIAamp® DNA Stool Mini Kit resulted in a lower DNA concentration. Heat treatment of oocysts/sporocysts before DNA isolation did not seem to improve the DNA yield, but only increased the duration of DNA isolation. There was no difference in DNA yield from sporocysts/oocysts between those that had been stored in water or ethanol before DNA isolation.

Specificity of the specific primer pairs and sensitivity of the PCR assay. Primer combination SD1f/SD1r produced a single band of approximately 620 bp on the agarose gels for *S. alces*. This primer combination did not amplify any other DNA tested in the PCR assay. Primer combination SD2f/SD1r produced a band of approximately 400 bp on the agarose gels and was specific for *S. alces*. Primer set SD3f/SD3r produced a band of approximately 680 bp on agarose gels for both *S. ovalis* and *S. hardangeri*. No PCR product was produced for any other DNA tested in the assay.

PCR products from DNA templates diluted 1 : 100 (or 0·01 ng/μl of *S. alces* and 0·13 ng/μl of *S. ovalis*) were still clearly visible on agarose gels after amplification using any of the 3 above-mentioned primer combinations. The sensitivity of the primers was not tested at further dilutions.

Experiment 1. Samples from 7 of the 8 foxes were positive on agarose gels after PCR using general coccidian primers. Samples from 3 foxes were positive on agarose gels after PCR using *S. alces*-specific primers. Samples from 7 animals were positive on agarose gels after PCR using *Hammondia*-specific primers. All PCRs using *S. ovalis*-specific primers and *Neospora*-specific primers were negative. The *Neospora*-specific primers were initially evaluated using DNA from *S. alces, S. hjorti*, and *Hammondia* sp., and all of these PCRs were negative as expected.

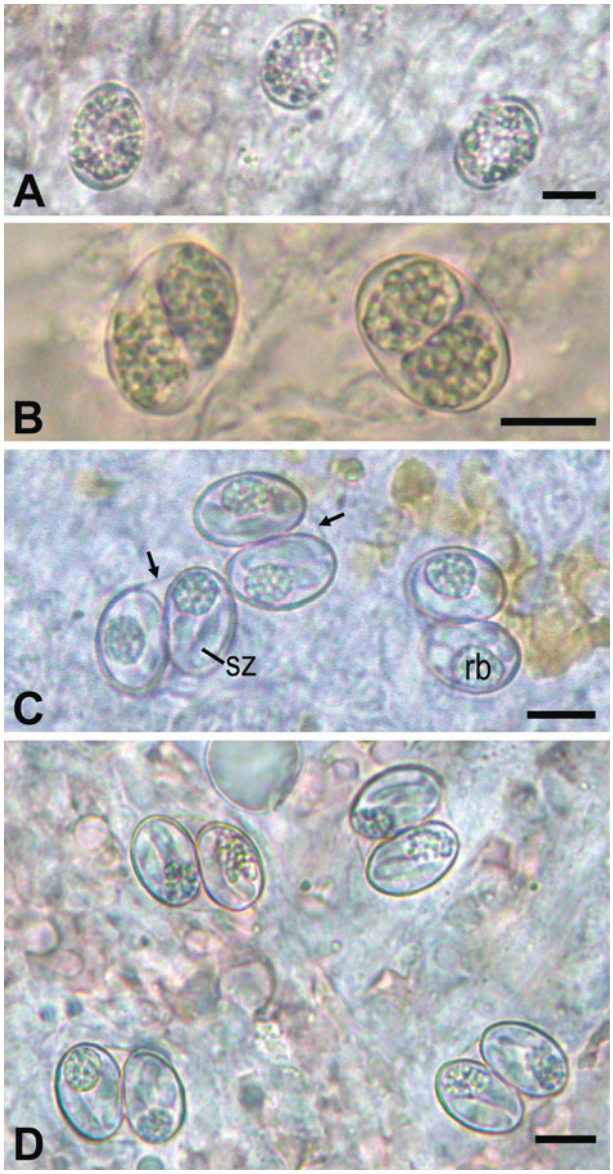

Fig. 3. Oocysts of *Sarcocystis alces* and/or *S. hjorti* in scrapings from the intestinal mucosa of foxes fed muscle tissues of moose. (A) Unsporulated oocysts in a blue fox 7 days post-infection. (B) Partly sporulated oocysts in a blue fox 8 days post-infection. (C) Fully sporulated oocysts in a silver (red) fox 14 days post-infection. The oocysts have a thin wall (arrows) and each oocyst contains 2 sporocysts with 4 sporozoites (sz) and a granular residual body (rb). (D) Sporulated oocysts in a silver fox 15 days post-infection. Scale bars = 10 μm in (A – D).

One sample from each of the 7 foxes that had been positive for coccidian DNA using the universal primers was sequenced. For most PCR products, fine single peak sequencing-chromatograms indicated the presence of DNA from either of *S. alces, S. hjorti* or *Hammondia* sp. However, sequencing reactions of a few other samples resulted in multiple peaks on chromatograms from 1 of the 2 sequencing primers (forward or reverse), or the sequencing primers

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produced 2 different DNA sequences. In total, 3 of the foxes were proven by sequence analysis to be infected by *S. alces* and 2 foxes by *S. hjorti* (Table 2).

Experiment 2. All 4 foxes were positive on agarose gels after PCR using general coccidian primers, whereas 1 fox was positive on agarose gel after PCR using the *S. alces*-specific primers. All PCRs using *S. ovalis*-specific primers, *Hammondia*-specific primers, or *Neospora*-specific primers were negative.

One sample from each of the 4 foxes which had been positive for coccidian DNA using the universal primers was sequenced. Most sequencing reactions resulted in fine single peak chromatograms which indicated the presence of DNA from either *S. alces* or *S. hjorti*, but a few samples resulted in multiple peaks on the chromatogram for one of the sequencing primers (forward or reverse), or the sequencing primers produced 2 different DNA sequences. Sequence analysis indicated that all 4 foxes were infected with *S. hjorti* and at least 1 fox also with *S. alces* (Table 2).

Examination of muscle samples of moose in 2009 for sarcocysts of S. hjorti

In 4 of 11 moose examined, altogether 7 cysts were consistent with those of *S. hjorti*, i.e., they were small, slender, and thread-like, measuring about $2 \times 0{\cdot}2$ mm, with about 10 μm long, hair-like protrusions (Fig. 1C). The surrounding host cell material was difficult to separate from these cysts and some of the hair-like protrusions were torn off during this process, or only parts of the cyst surface were exposed. *S. hjorti*-like cysts were only found in the diaphragm. All 11 moose examined, had numerous cysts consistent with those of *S. alces*, i.e., they were spindle-shaped, approximately $1–4 \times 0{\cdot}2–0{\cdot}3$ mm, with no visible surface protrusions, or with barely visible knob-like protrusions. Ten moose also had large oval sarcocysts consistent with those of *S. ovalis* (Dahlgren and Gjerde, 2008).

DISCUSSION

The present study revealed that red foxes and arctic foxes may serve as definitive hosts for 2 *Sarcocystis* species, i.e., *S. alces* and *S. hjorti*, as well as 1 *Hammondia* sp., using moose as intermediate host. The foxes were not suitable definitive hosts for the third *Sarcocystis* species, i.e. S. *ovalis*, present in the moose musculature used in the experiments. Such mixed natural infections with several *Sarcocystis* species, using different definitive hosts, are very common both in domestic animals (Dubey *et al.* 1989) and in free-ranging cervids (Dahlgren and Gjerde, 2007, 2008, 2009, 2010). By microscopy, it was only possible to differentiate between *Hammondia*-type oocysts and *Sarcocystis* oocysts, whereas molecular methods were necessary to further identify the *Sarcocystis* oocysts to species.

This seems to be the first study in which molecular methods have been used to verify the results of transmission experiments aimed at determining the definitive host(s) of *Sarcocystis* species of cervids. The life cycle of some species in cervids has been determined by feeding potential definitive hosts *Sarcocystis*-infected muscle tissue followed by screening of the faeces and/or the intestinal mucosa of the carnivores for oocysts/sporocysts, and then simply assuming that the sporocysts belonged to the (single) species identified as sarcocysts in the intermediate host (e.g., *S. alceslatrans* in moose: Dubey, 1980; Colwell and Mahrt, 1983). In a few experiments, micro-isolated sarcocysts of a single species have been used to ascertain that the sporocysts belonged to a particular species (e.g., *S. rangi* of reindeer: Gjerde, 1985*a*), or the identity of the sporocysts has been verified by their ability to induce formation of sarcocysts consistent with particular species after inoculation into intermediate hosts (e.g., *S. gracilis* and *S. capreolicanis* in roe deer: Erber *et al.* 1978). The oocysts/sporocysts of only a few *Sarcocystis* species, particularly those of *S. neurona* and *S. falcatula* in opossums, have previously been identified by molecular methods (Fenger *et al.* 1995; Tanhauser *et al.* 1999; Cheadle *et al.* 2001; Elsheikha *et al.* 2004; Xiang *et al.* 2009).

In the present study, molecular methods proved to be crucial in detecting a mixed infection with *S. alces* and *S. hjorti* in the foxes, and hence in the moose used as a source of the infective feed. Surprisingly, more foxes appeared to be infected with *S. hjorti* than with *S. alces*, even though the moose musculature used in both experiments seemed to contain mainly cysts of *S. alces*. An examination of muscle samples from an additional 11 moose subsequent to the infection experiments, with this new knowledge in mind, confirmed that cysts of *S. alces* vastly outnumbered those of *S. hjorti* in moose. The more frequent finding of *S. hjorti* in the foxes could be valid and, if so, was possibly due to a higher infectivity of *S. hjorti* than of *S. alces*, either as an inherent property of the species, or related to a better survival of the cysts after the death of the host, or it could be an artefact of the molecular methods used.

In the present study, sarcocysts of *S. hjorti* were described for the first time from moose. The small size and scarcity of sarcocysts of *S. hjorti* in moose compared with those of *S. alces*, may explain why no cysts of *S. hjorti* were detected by microscopy in a previous study of *Sarcocystis* species in Norwegian moose, although 1 cyst of *S. hjorti* (referred to as *Sarcocystis* sp. Type E) was detected by molecular methods (Dahlgren and Gjerde, 2008). The seemingly higher prevalence and infection intensity of *S. hjorti* in red deer as compared with moose, suggest that this species is better adapted to red deer than to

moose. This study also confirmed that sarcocysts of *S. hjorti* in Norwegian moose are morphologically very similar to those of *S. alceslatrans* previously described from Canadian moose (Dahlgren and Gjerde, 2008), which had been expected from the appearance of *S. hjorti* in red deer and the close phylogenetic relationship between *S. hjorti* and *S. alceslatrans* (Dahlgren and Gjerde, 2010).

Based on their phylogenetic position (Dahlgren and Gjerde, 2008, 2010), we had predicted that *S. alces* and *S. hjorti* would use canids as definitive hosts, and these predictions were confirmed in the present study. Previous studies by others have also indicated that different *Sarcocystis* species co-evolved with their definitive hosts (Holmdahl *et al.* 1999; Doležel, 1999; Slapeta *et al.* 2003; Elsheikha *et al.* 2005), and phylogenetic analysis may therefore be a useful complementary tool in the search for the definitive host(s) of those *Sarcocystis* species for which they are unknown (Dahlgren *et al.* 2008). Presumably, dogs and other canids may also act as definitive hosts for *S. alces* and *S. hjorti* in addition to foxes. Since *S. alceslatrans* has previously been found to use dogs and coyotes as definitive hosts (Dubey, 1980; Colwell and Mahrt, 1983), there are currently 3 known *Sarcocystis* species in moose that are being transmitted by canids. However, there might be still another species in moose using canids as definitive hosts. As noted previously (Dahlgren and Gjerde, 2008), Sedlaczek and Zipper (1986) described, by use of transmission electron microscopy, sarcocysts of a species in a moose in Germany, which they believed were *S. alceslatrans*, but which are more similar to *S. grueneri* of reindeer (Gjerde, 1985*b*), *S. cervicanis* of European red deer (Hernández *et al.* 1981) and *S. wapiti* of North American wapiti (Speer and Dubey, 1982), all of which use canids as definitive hosts.

In this study, sensitive primer pairs were developed, which specifically amplify parts of the ssu rRNA gene of either *S. alces* or *S. hardangeri* and *S. ovalis*. DNA from *S. hardangeri*/*S. ovalis* was not detected in the faeces or intestinal mucosa of any fox, and hence canids are apparently not suitable definitive hosts for these two species, which is not very likely based on their phylogenetic positions (Dahlgren and Gjerde, 2010). The use of species-specific primers provides a fast and easy method for screening multiple samples for a particular *Sarcocystis* species. However, as seen from this study, it is necessary to use more general primers and sequence a few samples in order to detect a mixed infection with unexpected species, not targeted by species-specific primers.

In Experiment 1, all foxes shed or harboured *Hammondia heydorni*-like oocysts. PCR-assays using *Hammondia*-specific and *Neospora caninum*-specific primers, confirmed that these oocysts belonged to a *Hammondia* species, and not to the morphologically indistinguishable species *N. caninum*, which, however, does not seem to use foxes as definitive hosts (Dubey *et al.* 2002; Schares *et al.* 2002). Recent molecular comparisons of *Hammondia* isolates from foxes and dogs, respectively, have indicated that they are different at the D2/D3 domain of the large subunit rRNA gene and in the intron 1 region of the alpha tubulin gene (Schares *et al.* 2002; Mohammed *et al.* 2003; Abel *et al.* 2006), and that *Hammondia* sp. of foxes might therefore be different from *Hammondia heydorni* of dogs. Hence, it will be of considerable interest to examine the present *Hammondia* isolate from foxes more extensively at various genetic markers to determine its relationship to other *Hammondia* isolates from foxes and dogs. Moose has previously been reported to be an intermediate host of *H. heydorni* infecting dogs (Dubey and Williams, 1980), but this seems to be the first report of moose as intermediate hosts of a *Hammondia* sp. using foxes as definitive hosts. Previously, sheep (Ashford, 1977), roe deer (Entzeroth *et al.* 1978), reindeer (Gjerde, 1983), sheep and goats (Schares *et al.* 2002) and the Arabian mountain gazelle (Mohammed *et al.* 2003) have been reported to be natural intermediate hosts of a *Hammondia* sp. infecting foxes. The oocyst morphology, pre-patent period and pattern of oocyst shedding in the foxes in the present study were similar to those reported from *Hammondia* sp. in foxes in the above-mentioned papers, but also similar to those of *H. heydorni* in dogs (summarized in Table 2 in Dubey *et al.* 2002).

In Experiment 1, several blue foxes and silver foxes developed reduced feed intake or gastrointestinal symptoms of 1 to a few days duration, about 1 week after infection. These symptoms were most likely associated with the *Hammondia* infection. Thus, *H. heydorni* infections in dogs have been associated with diarrhoea (Abel *et al.* 2006). The intestinal development of the 2 *Sarcocystis* species and the *Hammondia* sp. did not seem to affect any of the blood parameters analysed in this study. It is possible that the infection intensity was too low to produce any recognizable changes in the blood parameters, or that infection with *Sarcoystis* or *Hammondia* only caused local damage to the intestinal mucosa, without affecting any component of the blood. *Sarcocystis* species are generally considered to be non-pathogenic for their definitive hosts (Dubey *et al.* 1989).

In conclusion, the definitive host of *S. alces* and *S. hjorti* was determined by experimental infection and molecular diagnosis to be the red fox and the arctic fox. The first experiment also showed that moose may act as intermediate host for a *Hammondia* species using foxes as definitive hosts, but further molecular studies are necessary in order to determine whether this *Hammondia* isolate should be regarded as a species different from *H. heydorni* of dogs.

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