

# *Hammondia truffittae* n. comb. of foxes (*Vulpes* spp.): biological and molecular characteristics and differentiation from *Hammondia heydorni* of dogs

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## SUMMARY

Genomic DNAs from 3 oocyst isolates of *Hammondia* sp. from foxes (*Vulpes vulpes* and *V. lagopus*) and 1 oocyst isolate of *Hammondia heydorni* from a dog, were examined by PCR and sequence analysis of 6 loci in order to determine whether the isolates were conspecific. Consistent genetic differences were found between the fox and dog isolates, respectively, at the ITS-2 region, the *lsu* rRNA gene, the alpha tubulin gene and the HSP70 gene, but not at the *ssu* rRNA gene or ITS-1 locus. Infection experiments established that dogs were unsuitable as definitive hosts for *Hammondia* sp. of foxes; hence this species is regarded as separate from *H. heydorni* of wolf-like canids, but probably identical with *Isospora truffittae* (syn. *Isospora truffitti*) previously reported from foxes. This species has therefore been named *Hammondia truffittae* n. comb. Reindeer, moose, sheep, goats, foxes and rabbits may act as intermediate hosts for *H. truffittae*. Muscle tissues of inoculated intermediate hosts were infectious for foxes from 16 days post-infection. Oocysts of *H. truffittae* were subspherical, averaging  $12.5 \times 10.9 \mu\text{m}$  in size. The *lsu* rRNA gene and the alpha tubulin gene seem to be suitable genetic markers for differentiating between *H. truffittae* and *H. heydorni*.

Key words: *Hammondia truffittae*, *Hammondia heydorni*, foxes, dogs, life cycle, molecular characterization.

## INTRODUCTION

The genus *Hammondia* was erected to accommodate the newly discovered *Toxoplasma gondii*-like, but obligatory heteroxenous coccidian *Hammondia hammondi*, using cats as definitive hosts (Frenkel and Dubey, 1975). The oocysts of *H. hammondi* were morphologically indistinguishable from those of *T. gondii* of cats, as well as from oocysts of the small race or type of *Isospora bigemina*, which had originally been reported from dogs by Wenyon and Sheather (1925) and Wenyon (1926). Heydorn (1973) and Fayer (1974) established that the small race of *I. bigemina* had a two-host life cycle, using dogs as definitive hosts and cattle as intermediate hosts. Other ruminants and coyotes were soon found to act as additional intermediate and definitive hosts, respectively, for this species (e.g., Dubey and Fayer, 1976; Dissanaik and Kan, 1977; Dubey and Williams, 1980; Nassar *et al.* 1983; Warrag and Hussain, 1983). *Isospora bigemina* in dogs was re-named *Isospora heydorni* by Tadros and Laarman (1976) and transferred to the genus *Hammondia* by Dubey (1977); hence the currently used name *Hammondia heydorni*. In 1998, McAllister *et al.* reported that dogs also acted as definitive host for

the pathogenic species *Neospora caninum*, which possessed oocysts that were morphologically indistinguishable from those of *H. heydorni*. From now on, it became epidemiologically important to accurately identify this type of oocyst in dogs, and molecular methods were developed for this purpose (Šlapeta *et al.* 2002a, b).

Oocysts similar in size and shape to those of *H. heydorni* in dogs have also been found in the feces of foxes in several studies, as summarized in Table 1. These studies have included surveys for intestinal parasites of naturally infected free-ranging or farm-raised foxes, as well as experimental infections of foxes with muscle tissues of various hosts, mainly in order to elucidate the life cycle of various *Sarcocystis* species. Thus, in Norway, *Hammondia heydorni*-like oocysts were initially detected in foxes used in experiments to determine their role as definitive hosts for *Sarcocystis* species of reindeer (Gjerde, 1983), and more recently in foxes fed muscle tissues of *Sarcocystis*-infected moose (Dahlgren and Gjerde, 2010). As seen from Table 1, various species designations have been assigned to the *H. heydorni*-like oocysts in foxes, and most authors have believed them to be conspecific with *I. bigemina*/*H. heydorni* of dogs. However, Nukerbaeva and Svanbaev (1973) believed that various *Isospora* species were host specific, and named the *I. bigemina*-like oocysts they found in farmed silver and arctic foxes in Kazakhstan, *Isospora*

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Table 1. Overview of previous studies detecting *Hammondia*-like oocysts in faecal samples from foxes

Investigators and references	Country	Type of study (parasites studied)	Source of infection for foxes	Number and type of foxes shedding oocysts	Oocyst size	Species designation used
Triffitt (1927)	Canada	Diagnostic samples	Unknown; natural infection	Unclear; probably more than one fox; farm-raised	12.5–14.0 × 11.5–13.0 μm	<i>Isospora bigemina</i> -like
Lee (1934)	USA	Diagnostic samples; experimental infections using oocysts	Unknown, natural infection (possibly horse meat used as feed)	Composite fecal sample from farm-raised silver foxes; also in 2 foxes fed similar oocysts from dogs	12–17 × 9–11 μm	<i>Isospora bigemina</i> -like
Nukerbaeva and Svanbaev (1973)	Kazakhstan	Survey of intestinal parasites	Unknown; natural infection	17 of 1199 silver foxes ( <i>Vulpes fulva</i> ) and in some arctic foxes on fur farms	11–13 μm or 11–13 × 10–11 μm	<i>Isospora truffitti</i> n. sp.
Golemanski and Ridzhakov (1975)	Bulgaria	Survey of intestinal parasites	Unknown; natural infection	5 of 146 free-ranging red foxes ( <i>Vulpes vulpes</i> )	12.7–17.8 × 10.2–15.2 μm	<i>Isospora bigemina</i>
Ashford (1977)	UK	Exp. infections with muscle tissues ( <i>Sarcocystis</i> )	Naturally infected sheep ( <i>Ovis aries</i> )	1 of 5 red foxes (captured wild foxes used in experiments)	14 × 12 μm	<i>Hammondia</i> sp.
Entzeroth <i>et al.</i> (1978)	Germany	Exp. infections with muscle tissues (incl. <i>Sarcocystis</i> )	Naturally infected roe deer ( <i>Capreolus capreolus</i> )	1 of 1 fox (but not a dog and a cat)	13.1 × 11.6 μm	<i>Hammondia hammondi</i>
Gjerde and Helle (1982); Gjerde (1983)	Norway	Exp. infections with muscle tissues ( <i>Sarcocystis</i> )	Naturally infected reindeer ( <i>Rangifer tarandus</i> )	8 of 12 foxes (4 <i>Vulpes vulpes</i> , 4 <i>V. lagopus</i> ; farm-raised), but none of 6 dogs	10.7–15.3 × 9.7–12.8 μm	<i>Hammondia</i> sp., <i>H. heydorni</i> -like
Dubey (1982)	USA	Survey of intestinal parasites	Unknown; natural infection	1 of 198 free-ranging red foxes	Not given	<i>Hammondia heydorni</i> -like
Schares <i>et al.</i> (2002); Abel <i>et al.</i> (2006)	Germany	Exp. infections with muscle tissues of a sheep and a goat ( <i>Neospora caninum</i> )	Naturally infected sheep and goat; experimentally infected guinea pig	Sheep: 3 of 3 foxes and 0 of 2 dogs. Goat: 2 of 2 foxes, 0 of 3 dogs. Guinea pig: 1 of 1 fox. Farm-raised foxes	11.6–13.7 × 10.9–12.9 μm	<i>Hammondia</i> sp.
Ellis <i>et al.</i> (1999); Mohammed <i>et al.</i> (2003); Abel <i>et al.</i> (2006)	Saudi Arabia	Exp. infections with muscle tissues ( <i>Sarcocystis</i> )	Naturally infected Arabian mountain gazelle ( <i>Gazella gazella</i> )	1 of 2 Arabian red foxes ( <i>Vulpes vulpes arabica</i> ); foxes obtained from a zoo	10.9 × 10.1 μm	<i>Hammondia heydorni</i>
Gerasimchik (2005)	Belarus	Survey of intestinal parasites	Unknown; natural infection	6 of 1625 silver foxes ( <i>Vulpes vulpes fulva</i> ) on 5 fur farms	11.5–13.3 μm	<i>Isospora truffitti</i>
Gerasimchik (2006)	Belarus	Survey of intestinal parasites	Unknown; natural infection	18 of 3168 arctic foxes ( <i>Vulpes lagopus</i> ) on 7 fur farms	Not given	<i>Isospora truffitti</i>
Gjerde (unpublished)	Norway	Survey of intestinal parasites (2002–04)	Unknown; natural infection	2 of 271 wild red foxes	About 12 μm	<i>Hammondia</i> sp.
Dahlgren and Gjerde (2010)	Norway	Exp. infections with muscle tissues ( <i>Sarcocystis</i> )	Naturally infected moose ( <i>Alces alces</i> )	8 of 8 foxes (4 silver and 4 blue foxes; farm-raised)	10–12 μm	<i>Hammondia</i> sp.

*triffitti* n. sp. in recognition of Marjorie J. Triffitt, who had first described such oocysts from foxes (Triffitt, 1927). This species name was later amended to *Isospora triffittae* by Levine (1985), since the species had been named after a woman.

Some infection experiments have suggested that the *Hammondia* sp. infecting foxes is not infectious for dogs (Enzeroth *et al.* 1978; Gjerde, 1983; Schares *et al.* 2002). Similarly, molecular comparisons at a few loci between fox-derived *Hammondia* isolates and *H. heydorni* isolates from dogs, have indicated that they may represent 2 distinct genetic lineages (Schaes *et al.* 2002; Mohammed *et al.* 2003; Abel *et al.* 2006). Since molecular and biological data from more *Hammondia* isolates from foxes might help resolve the question of whether *Hammondia* sp. in foxes is different from *Hammondia heydorni* of dogs, the present study was initiated after having been prompted by the unexpected finding of *Hammondia* oocysts in foxes fed muscle tissues of moose (Dahlgren and Gjerde, 2010). In addition to this recent *Hammondia* isolate from foxes, we had available 2 similar, about 25-year-old oocyst isolates from foxes, derived directly, or after passage through sheep, from naturally infected reindeer (Gjerde, 1983), as well an isolate of *H. heydorni*-like oocysts from a naturally infected Norwegian dog.

Moreover, a considerable amount of biological and morphological data about the *Hammondia* sp. of foxes had been gathered during a series of experiments conducted in 1982–1984, subsequent to the initial finding of this coccidian in foxes in Norway in 1981 (Gjerde, 1983). The major aims of these experiments were to elucidate the biology of this *Hammondia* sp., and particularly to determine: (1) its development in foxes, including the length of pre-patent and patent periods, the pattern of oocyst shedding and possible variations in oocyst morphology during patency; (2) whether carnivores other than foxes, particularly dogs, might act as definitive hosts; (3) the suitability of foxes, sheep, goats, rabbits and cats as intermediate hosts, including the timing of the formation of the infective stage in these hosts post-infection. The results of most of these studies have, however, not been published before, since it could not be conclusively determined whether the *Hammondia* sp. of foxes was different from *H. heydorni* in dogs, which already had been the topic of several similar studies by others. Since current molecular methods have the potential to resolve this crucial question, the major aims of the molecular examination of the 3 fox-derived and 1 dog-derived oocyst isolates were: (1) to PCR-amplify and sequence up to 6 genetic markers from each of the isolates; (2) to compare the new sequences with each other and with corresponding sequences available in GenBank of *H. heydorni*-like isolates from foxes and dogs in other countries; (3) based on this comparison, and the morphological and biological data obtained from the infection

experiments, decide whether *Hammondia* sp. of foxes should be considered a separate species or a variant of *H. heydorni*.

For the reasons stated and discussed subsequently, *Hammondia* sp. of true foxes is considered to represent a separate species, closely related to, but different from, *H. heydorni* of dogs, but probably identical to *I. triffitti* of foxes as described by Nukerbaeva and Svanbaev (1973). The name *Hammondia triffittae* has therefore been used in the following.

#### MATERIALS AND METHODS

##### *Infection experiments with reindeer-fox isolates of Hammondia triffittae*

The general design and outcome of experiments F1–F16, which comprised the feeding of foxes and other carnivores with muscle tissues of naturally infected reindeer and potential intermediate hosts experimentally inoculated with *Hammondia* oocysts, are presented in Table 2. Below are some additional data that were common to all or most of these experiments, as well as some details about the experimental animals used.

*Potential definitive hosts fed muscle tissues.* All silver foxes (colour mutants of the red fox, *Vulpes vulpes*), blue foxes (colour mutants of the arctic fox, *Vulpes (=Alopex) lagopus*), raccoon dogs (*Nyctereutes procyonoides*), and minks (*Mustela vison*) had been born and raised at the Dal Research Farm for Fur-bearing Animals, at Heggedal, near Oslo, and were kept at this facility during the experiments. All dogs and cats were kept at different clinics at the Norwegian School of Veterinary Science (NVH), Oslo, during the experiments. None of the experimental animals, except possibly the cats, had eaten raw musculature prior to the experiments. During the experimental periods, the foxes, raccoon dogs and minks were fed the usual fox feed at the Research Farm, consisting mainly of heat-treated slaughterhouse offal, fish offal and cereals. The dogs and cats were fed commercial dry or canned dog and cat feed, respectively. The animals' ordinary feed was usually withheld on the day of experimental feeding with muscle tissues.

*Foxes.* A total of 55 foxes were used in 16 separate experiments (F1–F16). Forty-six foxes were fed fresh muscle tissues, 4 foxes were fed muscle tissues that had been kept frozen for 2 or 5 days, and 5 foxes were used as uninfected controls. The foxes were kept individually in wire-mesh cages in open sheds, and fecal samples were usually collected daily from the ground below the cages and placed in labelled plastic bags. In most experiments, both silver and blue foxes were used (Table 2). In total, 39 blue foxes (*Vl*), 15 silver foxes (*Vs*), and 1 silver and blue fox hybrid

Table 2. Design and outcome of experimental infections of foxes (*Vv*=silver/red foxes; *Vl*=blue/arctic foxes) and other potential definitive hosts with muscle tissues of animals naturally or experimentally infected with oocysts of *Hammondia triffittae*

Exp. no.	Origin and nature of muscle tissues fed to potential definitive hosts	Animals being fed muscle tissues	Days with fecal examination post-infection	Days post-infection (dpi) with shedding of <i>Hammondia</i> oocysts (many oocysts) and [ <i>Sarcocystis</i> sporocysts] <sup>1</sup>
F1	Naturally infected reindeer (Rt1), slaughtered 10.02.81; muscles fed to 4 foxes and 2 dogs on 12.02.81 (fresh) or on 17.12.81 (kept frozen at -20 °C for 5 days, Fz-5d)	Fox 1 ( <i>Vl</i> )	-1, 0, 6-69	7-20 (7-9) dpi; [Sa: 15-46 dpi]
		Fox 2 ( <i>Vl</i> )	-1, 0, 6-69	7-25 (7-9) dpi; [Sa: 16-41 dpi]
		Fox 3 ( <i>Vv</i> )	-1, 0, 6-69	7-29 (9) dpi; [Sa: 15-56 dpi]
		Fox 4 ( <i>Vv</i> )	-1, 0, 6-69	7-25 (7, 10) dpi; [Sa: 16-31 dpi]
		Fox 5 ( <i>Vl</i> ) (Fz-5d)	-1, 0, 6-69	None
		Fox 6 ( <i>Vv</i> ) (Fz-5d)	-1, 0, 6-69	None
		Fox 7 ( <i>Vl</i> ) (Control)	-1, 0, 6-69	None
		Fox 8 ( <i>Vv</i> ) (Control)	-1, 0, 6-69	None
		Dog 1	0-4, 11-16†	None
		Dog 2	0-4, 11-39	None
		Dog 3 (Fz-5d)	0-4, 11-39	None
Dog 4 (Control)	0-4, 11-39	None		
F2	Naturally infected reindeer (Rt2), slaughtered 02.12.81; fresh muscles fed to foxes 04.12.81	Fox 9 ( <i>Vl</i> )	1-33	7-12 (7-9) dpi
		Fox 10 ( <i>Vv</i> )	1-33	7-18 (8-11) dpi; [Sa: 17-33 dpi]
		Fox 11 ( <i>Vl</i> ) (Control)	1-33	None
		Fox 12 ( <i>Vv</i> ) (Control)	1-33	None
F3	Two naturally infected reindeer (Rt3, Rt4) slaughtered 04.03.82; muscles fed to foxes and dogs 06.03.82 (fresh) or 11.03.82 (kept frozen at -20 °C for 2 days, Fz-2d)	Fox 13 ( <i>Vl</i> )	2-24	7-14 (8-9) dpi; [Sa: 17-24 dpi]
		Fox 14 ( <i>Vv</i> )	2-24	7-22 (8-9) dpi; [Sa: 17-23 dpi]
		Fox 15 ( <i>Vl</i> ) (Fz-2d)	7-19	None
		Fox 16 ( <i>Vv</i> ) (Fz-2d)	7-19	None
		Dog 5	2-19	None; [Sa: 17-19 dpi]
		Dog 6	2-19	None; [Sa: 16-19 dpi]
F4	Lamb (Sh1) inoculated with oocysts from Foxes 13 and 14 in F3; killed 51 dpi (19.08.83); muscles fed to foxes 19.-20.08.82	Fox 17 ( <i>Vl</i> )	0-18	4-15 (4-8) dpi
		Fox 18 ( <i>Vl</i> )	0-18	4-14 (5-9) dpi
		Fox 19 ( <i>Vv</i> )	0-18	4-13 (4-8) dpi
		Fox 20 ( <i>Vv</i> )	0-18	4-15 (4-12) dpi
F5	Lamb (Sh2) inoculated with oocysts from Foxes 13 and 14; killed 105 dpi (12.10.82); muscles fed to foxes, dogs and minks 12.10.82 (to dog 7 also on 13.10.82)	Fox 21 ( <i>Vl</i> )	2-17	None
		Fox 22 ( <i>Vl</i> )	2-17	4-13 (4-8) dpi
		Fox 23 ( <i>Vl</i> )	2-17	3-11 (9) dpi
		Fox 24 ( <i>Vl</i> )	2-17	4-15 (6-10) dpi
		Fox 25 ( <i>Vl</i> )	2-17	4-16 (6-10) dpi
		Fox 26 ( <i>Vv</i> ) (Control)	2-17	None
		Dog 7	4-15	None
		Dog 8	4-12	None
		Dog 9	4-12	None
		Minks 1 and 2	2-14	None
		Minks 3 and 4	2-14	None
	Uninoculated ewe (Sh3), kept with Sh1 and Sh2; slaughtered 12.10.82; muscles fed to a fox 12.10.82	Fox 27 ( <i>Vl</i> )	2-31	6-14 (8-9) dpi; [Sa: 8-31 dpi]

F6	Two blue foxes inoculated with oocysts from Foxes 22–25 in F5; killed 20 dpi (02.12.82); muscles fed to foxes 02.–03.12.82 Uninoculated blue fox killed 14.12.82; muscles fed to a fox 14.–15.12.82	Fox 28 ( <i>Vl</i> )	0–25	6–25 (6–10) dpi
		Fox 29 ( <i>Vl</i> )	0–25	5–13 (5–10) dpi
		Fox 30 ( <i>Vl</i> )	2–13	None
F7	Eight naturally infected reindeer (Rt5–Rt12) slaughtered 02.03.83; cardiac (CaM) or skeletal muscles (SkM) fed to foxes 07.03.83	Fox 31 ( <i>Vl</i> ) (CaM)	2–31	7–25 (7–11) dpi; [Sa: 10–31 dpi]
		Fox 32 ( <i>Vl</i> ) (CaM)	2–31	8–24 (8–13) dpi; [Sa: 14–31 dpi]
		Fox 33 ( <i>Vv</i> ) (SkM)	2–31	None; [Sa: 10–31 dpi]
		Fox 34 ( <i>Vv</i> ) (SkM)	2–31	None; [Sa: 10–28 dpi]
F8	Goat kid (Go1) inoculated with oocysts from Foxes 13 and 14; killed 16 dpi (17.03.83); muscles fed to a fox 17.–18.03.83	Fox 35 ( <i>Vl</i> )	2–28	5–19 (5–12) dpi
F9	Goat kid (Go2) inoculated with oocysts from Foxes 13 and 14; killed 44 dpi (14.04.83); muscles fed to foxes and a dog 14.04.83	Fox 36 ( <i>Vl</i> )	2–21	3–20 (4–9) dpi
		Dog 10	2–15	None
		Dog 11	2–15	None
F10	Uninoculated goat kid (Go5), killed 26.05.83; muscles fed to fox same day	Fox 37 ( <i>Vl</i> x <i>Vv</i> )	1–9 (1–21)	5 dpi
F11	Goat kid (Go3) inoculated with oocysts from Foxes 13 and 14; killed 107 dpi (16.06.83); muscles fed to a fox 16.08, and to dogs and cats 16.–18.06.83	Fox 38 ( <i>Vl</i> )	8 (0–22)	8 dpi
		Dog 10	4–15	None
		Dog 11	4–15	None
		Cats 1–3	4–13	None
F12	Goat kid (Go4) inoculated with oocysts from Foxes 13 and 14; killed 252 dpi (07.11.83); muscles fed to 6 foxes 07.11.83	Fox 39 ( <i>Vl</i> )	Killed 1 dpi	No stages seen
		Fox 40 ( <i>Vl</i> )	Killed 2 dpi	No stages seen
		Fox 41 ( <i>Vl</i> )	Killed 3 dpi	Schizonts in intestinal mucosa
		Fox 42 ( <i>Vl</i> )	Killed 4 dpi	A few oocysts in colon
		Fox 43 ( <i>Vl</i> )	Killed 5 dpi	Many oocysts in colon
		Fox 44 ( <i>Vl</i> )	Killed 9 dpi	Many oocysts in colon/faeces
	Uninoculated goat kid (Go6) killed 07.11.83; muscles fed to 6 foxes 07.11.83	Fox 45 ( <i>Vl</i> )	Killed 1 dpi	No stages seen
		Fox 46 ( <i>Vl</i> )	Killed 2 dpi	No stages seen
		Fox 47 ( <i>Vl</i> )	Killed 3 dpi	No stages seen
		Fox 48 ( <i>Vl</i> )	Killed 4 dpi	No stages seen
		Fox 49 ( <i>Vl</i> )	Killed 5 dpi	A few oocysts in colon
		Fox 50 ( <i>Vl</i> )	Killed 9 dpi	Some oocysts in colon/faeces
		Fox 51 ( <i>Vl</i> )	2–19	None
F13	Three cats (1–3) inoculated with oocysts from Foxes 13 and 14; killed 15 dpi (20.07.83); muscles fed to a fox 20.07.83			
F14	Lamb (Sh4) inoculated with oocysts from Foxes 44 and 50 in F12; killed 41 dpi (26.04.84); muscles fed to foxes and Dog 12 on 27.04.84; and to Dogs 13 and 14 and both raccoon dogs 27.–29.04.84	Fox 52 ( <i>Vv</i> )	1–13	5–12 (6) dpi
		Fox 53 ( <i>Vv</i> )	1–13	5–12 dpi
		Dog 12	4–13	None
		Dog 13	4–13	None
		Dog 14	4–13	None
		Raccoon dog 1	6	None
		Raccoon dog 2	6	None
F15	Rabbit (Ra1) inoculated with oocysts from Foxes 44, 50, 52 and 53 in F12 and F14; killed 40 dpi (03.07.84); muscles fed to fox 03.07.84	Fox 54 ( <i>Vl</i> )	6–13	6–11 (6–7) dpi
F16	Rabbit (Ra2) inoculated with oocysts from Foxes 44, 50, 52 and 53; killed 84 dpi (16.06.84); muscles fed to fox 16.08.84	Fox 55 ( <i>Vl</i> )	6–13	None

<sup>1</sup> The shedding/detection of *Sarcocystis* sporocysts was often intermittent during the given time period.

† Dog 1 euthanized 16 dpi due to severe diarrhoea caused by parvo virus infection.



(*Vv* × *Vl*) were used. The foxes were of both sexes and 6–24 months old at the time of infection.

**Raccoon dogs.** Two raccoon dogs (*Nyctereutes procyonoides*), about 1 year and 3 years of age, respectively, were used in Exp. F14. They were kept individually in 2 cages with wire-mesh floor, and fecal samples were collected from the ground below the cages.

**Minks.** Four minks (*Mustela vison*), about 16 months old, were used in Exp. F6. They were kept as pairs in 2 cages with wire-mesh floor, and fecal samples from each pair were collected from the ground below the cages.

**Dogs.** A total of 14 dogs were used in 6 separate experiments (F1, F5, F9–F11, F14). Twelve dogs were fed fresh muscle tissue, 1 dog was fed frozen and thawed muscle tissues, and 1 dog was an uninfected control. Dogs no. 10 and no. 11 were used in 2 separate experiments (F9 and F11). All dogs were obtained from a commercial breeder, rearing dogs for experimental purposes. They belonged to various breeds and ranged in age from about 6 weeks to about 6 months at the start of the experiments. The dogs were kept in individual cages, except the 6-week-old puppies used in Exp. F1, which were kept together with their mother for the first 10 days after the experimental feeding. Individual fecal samples were collected daily and examined for oocysts.

**Cats.** Three 6-week-old cats were used in experiment F11. These kittens were obtained from a litter on a farm shortly before the experiment, and were kept together in a single cage. Pooled fecal samples from all the cats were collected daily and examined for oocysts.

**Potential intermediate hosts.** Naturally infected reindeer and experimentally inoculated sheep, goats, foxes, cats and rabbits were evaluated as intermediate hosts by feeding muscle tissues from them to foxes upon euthanasia.

**Reindeer.** Muscle tissues used in Exps F1–F3 and F7 were obtained from adult reindeer slaughtered at an abattoir in Kautokeino, Finnmark County, Northern Norway. Muscle tissues (mostly abdominal muscles, diaphragm and heart) were collected from animals with numerous grossly visible sarcocysts in their skeletal muscles, since the initial purpose of these experiments was to study the infectivity of various *Sarcocystis* species of reindeer for foxes and dogs. The tissues were shipped to Oslo and fed to the experimental animals within 2–3 days after the reindeer had been slaughtered.

**Sheep.** Three lambs, which had been obtained from NVH's regular sheep flock, were used in 2 separate experiments. The 2 lambs used in Exps F4 and F5 had been born and raised indoors, and were kept in a pen at NVH together with their 3-year-old mother ewe throughout the experimental period. This ewe had been on pasture during 2 grazing seasons before the experiment. The lambs were about 2 months old when inoculated with oocysts. The single, 10-month-old lamb used in Exp. F14, had been kept indoors throughout its entire life, and was kept in a pen at NVH during the experimental period.

**Goats.** Six 1-week-old goat kids were obtained from Valbjør geiteavlsgård, Vågå (a research farm for goats), and were kept together in a single pen at NVH during the experimental period. Four of the goat kids were inoculated with oocysts when 2½ weeks old, while 2 kids served as inoculated controls. Muscle tissues from these goats were used in Exps F8–F12.

**Foxes.** Two adult blue foxes from Dal Research Farm were inoculated with oocysts, while a third blue fox from the farm served as an uninoculated control in Exp. F6. Fecal samples from both inoculated foxes were collected on days 6, 7 and 10 post-infection and examined for oocysts, to evaluate a possible direct life cycle.

**Cats.** The 3 cats fed muscle tissues from a goat kid in Exp. F11, were later inoculated with *Hammondia*-oocysts and subsequently euthanized. Muscle tissues from the cats were then fed to a fox in Exp. F13.

**Rabbits.** Two 6-month-old laboratory reared rabbits, kept in individual cages at the Research Animal Unit at NVH, were inoculated with oocysts and used in Exps F15 and F16.

**Sporulation of oocysts and administration of oocyst inocula.** Fecal samples from foxes, collected on days with high oocyst excretion, were suspended in 2.5% potassium dichromate, sieved through a coarse sieve, and left to sporulate in Petri dishes for several days at room temperature (22–24 °C). Sporulated oocysts were subsequently kept refrigerated in 2.5% potassium dichromate until used to infect animals. Before inoculation, oocysts were washed by repeated centrifugation in tap water to remove potassium dichromate. Lambs Sh1 and Sh2 each received about 10 000 sporulated oocysts, whereas the number of oocysts inoculated into other potential hosts was not accurately determined. In all experiments, oocysts from foxes were administered orally. Lambs, foxes and goat kids were inoculated through a stomach tube; rabbits were inoculated into the mouth using a syringe; and cats were given oocysts mixed with their ordinary feed. All animals were fed oocysts

once, except lamb Sh4, which received oocysts twice, 3 days apart.

The ability of *Hammondia* oocysts to sporulate after freezing was evaluated in a small experiment using fecal samples derived from foxes in Exp. F2. The samples were either kept for 3 weeks in the fridge at 4 °C; for 1 week in the fridge and 2 weeks in a freezer at -20 °C; or for 1 week in the fridge and 2 weeks outdoors at varying temperatures below zero. All samples were then suspended in 2.5% potassium dichromate in Petri dishes at room temperature and examined daily for sporulation of oocysts.

*Euthanasia of experimental animals.* Lambs, goats kids, cats and rabbits were euthanized with an overdose of pentobarbital (Mebumal 10%) either intravenously or intraperitoneally. The uninoculated control ewe (Sh3; F5) was slaughtered at an abattoir. Foxes used in Exps. F6 and F12 were killed by electrocution according to standard procedures at the research farm. The other foxes, as well as the raccoon dogs, minks and dogs, were only monitored for oocyst shedding, and were not killed.

*Preparation and administration of muscle tissue inocula.* Muscle tissues from the entire carcass (limbs, trunk, diaphragm and heart) of experimentally inoculated animals were removed, mixed well, cut into smaller pieces and ground. The muscle samples from 12 slaughtered reindeer and 1 slaughtered sheep were treated likewise, but in Exp. F7, the foxes were fed either skeletal or cardiac muscles from reindeer. In a few experiments, the foxes were fed tissues within 3–4 h after the inoculated animal had been killed; in the other experiments the ground muscle tissues were kept refrigerated overnight or for a few days before being fed to foxes, dogs or cats. In the initial experiments using reindeer muscles, some portions were kept frozen at -20 °C for 5 days (Exp. F1) or 2 days (Exp. F3) before being thawed and fed to foxes and dogs. About 300–500 g of muscle tissue were usually fed to each fox, raccoon dog or adult dog. Smaller portions were given to cats, minks and dog puppies. The animals were usually monitored for a short while after feeding to ascertain that they ingested most of the muscle tissues provided.

*Examination of fecal samples for oocysts.* The duration of the fecal examination of all foxes, dogs, raccoon dogs, minks and cats are given in Table 2. The extent of fecal examination varied somewhat between experiments for capacity reasons, but samples from days when the animals were expected to shed maximum numbers of oocysts, if infected, were always examined. Fecal samples were processed and examined for oocysts and sporocysts by microscopy at 200× magnification using a standard flotation technique with ZnCl<sub>2</sub>/NaCl-solution (specific gravity 1.3) as flotation fluid.

The size of unsporulated and sporulated oocysts and sporocysts was measured with a calibrated ocular screw micrometer. In order to determine whether oocyst size would change during patency, a fairly large number of oocysts from particular foxes were examined daily in some experiments. Thus, from Fox 31 in Exp. F7, 10–60 oocysts were measured from each of days 7–12 post-infection, as well as 20–40 oocysts from Fox 32 from each of days 8–12. Similarly, from Fox 35 in Exp. F8, 30 oocysts from each of days 5–8 post-infection were measured.

Black and white microphotographs of oocysts were recorded on film in connection with some of the experiments in 1981–1984, whereas digital colour photographs were taken in 2009 with a Leica DC480 digital camera of oocysts that had been kept refrigerated for up to 27 years.

#### *Hammondia triffittae oocysts obtained from foxes fed muscle tissues from moose*

In October 2007, 4 silver foxes and 4 blue foxes were fed *Sarcocystis*-infected muscle tissues from moose to determine whether they would act as definitive hosts for some of the *Sarcocystis* species of this intermediate host. All 8 foxes subsequently harboured or shed *Hammondia* oocysts and *Sarcocystis* oocysts/sporocysts as described previously (Exp. 1; Dahlgren and Gjerde, 2010). The *Hammondia* oocysts examined by molecular methods in the present study were collected from the intestinal contents and feces of a silver fox and a blue fox killed 7 days after being fed moose tissues. In both foxes, numerous unsporulated, subspherical *Hammondia* oocysts were observed in the mucosa along the entire posterior half of the small intestine. The oocysts were set to sporulate in 2.5% potassium dichromate at room temperature, and subsequently kept refrigerated for about a year until concentrated by sucrose flotation for molecular studies. Sporulated oocysts were measured with a calibrated ocular micrometer, and colour photographs of oocysts were recorded on film in 2007 and with a digital camera in 2010. Sporulated oocysts, when examined early in 2010, measured 11.3–13.8 × 10.8–12.8 μm (12.6 ± 0.5 × 11.9 ± 0.4), and their average length to width ratio was 1.06 ± 0.03 (1.00–1.13); n = 30.

#### *Hammondia heydorni oocysts obtained from a dog*

In August 2000, a veterinary clinic in Eastern Norway submitted a fecal sample from a 5-month-old female Rottweiler dog to the Parasitology lab at NVH for standard coprological examination for parasites, since the dog had suffered from gastrointestinal symptoms, including haemorrhagic diarrhoea, for about 4 weeks. The examination at the lab revealed numerous eggs of the hookworm *Uncinaria*

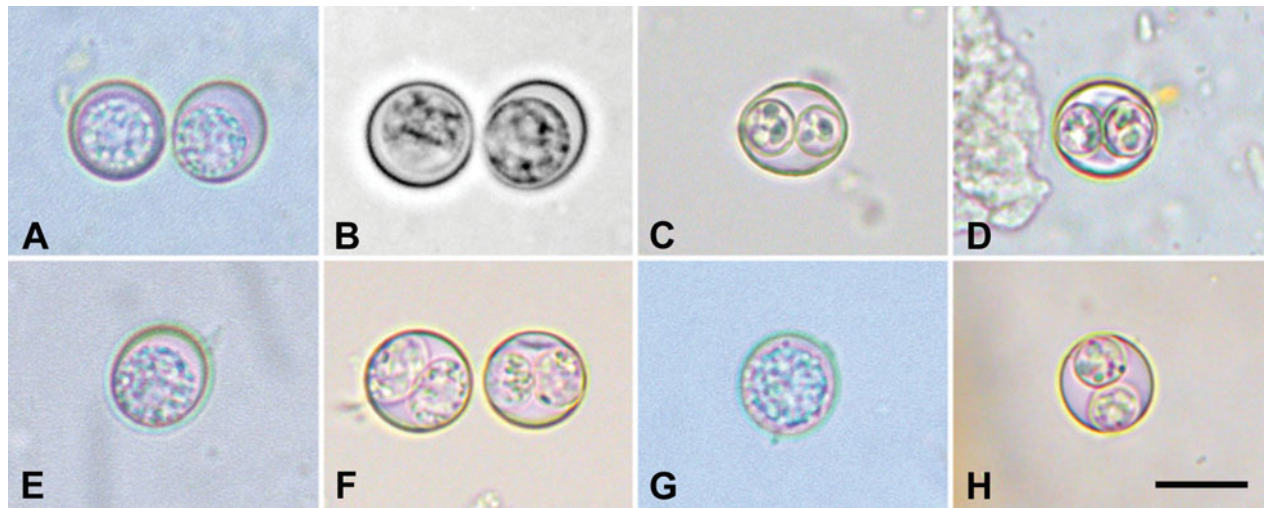


Fig. 1. Unsporulated and sporulated oocysts of *Hammondia triffittae* from foxes (A–F) and *Hammondia heydorni* from a dog (G–H). (A) Two unsporulated oocysts, presumably of *H. triffittae* (isolate not examined by molecular methods) from a free-ranging red fox killed in 2002. (B) Two unsporulated oocysts of *H. triffittae* from foxes fed reindeer meat in Exp. F2 in 1981. (C) A sporulated oocyst of the isolate shown in (B) after 27 years in a fridge. (D) A sporulated oocyst of *H. triffittae* from foxes fed muscles from an experimentally infected lamb in Exp. F5 in 1982 and stored for nearly 27 years in a fridge. (E–F) An unsporulated and 2 sporulated oocysts of *H. triffittae* from foxes fed moose musculature in 2007. (G–H) An unsporulated and a sporulated oocyst of *H. heydorni* from a naturally infected dog diagnosed in 2000; photo of sporulated oocyst after 9 years in a fridge. All photos shown at approximately the same magnification. Scale bar = 10  $\mu\text{m}$  for all photos.

*stenocephala*, a moderate number of *Cryptosporidium* oocysts, as well as a fairly large number of nearly spherical coccidian oocysts, measuring 11–13  $\mu\text{m}$  in diameter (Fig. 1G), consistent with those of *H. heydorni* and *N. caninum* of dogs. By request, fecal samples from 4 more days were submitted, 2 of which still contained a considerable number of *Hammondia/Neospora*-type oocysts. Fecal samples containing oocysts were suspended in 2.5% potassium dichromate, set to sporulate at room temperature, and sporulated oocysts were subsequently kept refrigerated for more than 8 years until used in the present investigation. Sporulated oocysts then measured 11.0–13.3  $\times$  10.3–12.5  $\mu\text{m}$  ( $12.2 \pm 0.7 \times 11.3 \pm 0.5 \mu\text{m}$ ), and their average length to width ratio was  $1.08 \pm (1.00–1.21)$  ( $n = 30$ ). Photographs of fresh oocysts were recorded on film in 2000, and additional photographs of stored oocysts were taken with a Leica DC480 digital camera in 2009.

#### Molecular analysis of 4 oocyst isolates: DNA extraction, PCR conditions and sequencing

**Oocyst isolates.** *Hammondia*-type oocysts from 4 isolates were genetically characterized by PCR and sequence analysis: (1) oocysts (Fig. 1C) obtained from foxes (*Vulpes vulpes* and *V. lagopus*; *Vulpes*) fed meat from naturally infected reindeer (*Rangifer tarandus*; Rt) in Exps F2 and F3 in 1981 (isolate *HtRtVulpes-1981*); (2) oocysts (Fig. 1D) obtained from foxes fed meat from an experimentally inoculated lamb (*Ovis aries*; Oa) in Exp. F5 in 1982 (isolate

*HtOaVulpes-1982*); (3) oocysts (Fig. 1F) obtained from foxes fed meat from naturally infected moose (*Alces alces*, Aa) in 2007 (isolate *HtAaVulpes-2007*); and (4) oocysts (Fig. 1H) isolated in year 2000 from a naturally infected dog, source of infection unknown (isolate *HhCanis-2000*).

Fecal suspensions of partly to fully sporulated oocysts of the above-mentioned isolates had been stored refrigerated in 2.5% potassium dichromate for 1 to 27 years. Before attempted DNA isolation, oocysts were washed by repeated centrifugation in tap water and then concentrated by flotation in saturated sucrose solution. Oocysts were either harvested directly from the top layer after flotation, or from the sediment following dilution and washing of the floated oocysts by repeated centrifugation in tap water. Isolated oocysts were kept frozen in microcentrifuge tubes at  $-20^\circ\text{C}$  for a few weeks before DNA extraction.

**DNA extraction.** The oocyst samples were frozen and thawed either once or thrice. After thawing, DNA was either extracted directly from the oocysts, or after incubation of a mixture of 100  $\mu\text{l}$  of the oocyst suspension and 150  $\mu\text{l}$  of Tris-EDTA buffer at  $100^\circ\text{C}$  for 60 min. In both cases, genomic DNA was extracted from the oocysts using QIAamp® DNA Mini Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions.

**Genetic markers and PCR conditions.** Six genomic DNA regions were PCR-amplified as detailed below.



In addition, genomic DNA extracted from isolate *HtAaVulpes-2007* was tested with the *N. caninum*-specific primer pair Np21/Np6 as described previously (Dahlgren and Gjerde, 2010).

**Ssu rRNA gene.** The complete ssu rRNA gene was amplified in 3 overlapping fragments, using primer pairs ERIB1/3Hr, S5f/S4r and S3f/Primer B, respectively, as previously described (Dahlgren and Gjerde, 2007).

**ITS-1.** The complete ITS-1 region was amplified using primer pair JB1/JB2 (Barratt *et al.* 2007). Cycling conditions were: initial Hot Start at 95 °C for 15 min, followed by 35 cycles of 94 °C for 40 s, 57 °C for 45 s, 72 °C for 1 min; and final extension at 72 °C for 10 min.

**ITS-2.** The complete ITS-2 region was amplified using primers Tim11F (Payne and Ellis, 1996) and T13Rmod (5'-CTACAAGTCAACAGCTCA-3'). Cycling conditions were: initial Hot Start at 95 °C for 15 min, followed by 35 cycles of 94 °C for 40 s, 55 °C for 45 s, 72 °C for 1 min; and final extension at 72 °C for 10 min.

**Lsu rRNA gene.** The D2/D3 domain of the lsu rRNA gene was amplified using primer pair CR1/CR2 (Ellis *et al.* 1999). Cycling conditions were: initial Hot Start at 95 °C for 15 min, followed by 35 cycles of 94 °C for 40 s, 55 °C for 45 s, 72 °C for 1 min; and final extension at 72 °C for 10 min.

**Alpha tubulin gene.** A small fragment (intron 1 region) of the alpha tubulin gene was amplified using primer pair AT264/AT9 (Abel *et al.* 2006). Cycling conditions were: initial Hot Start at 95 °C for 15 min, followed by 35 cycles of 94 °C for 40 s, 56 °C for 45 s, 72 °C for 1 min; and final extension at 72 °C for 10 min. Since different *Sarcocystis* species might occur concurrently with *Hammondia* in foxes, DNA from *Sarcocystis alces* and *Sarcocystis hjorti* was included as controls to test whether *Sarcocystis* DNA was also amplified. DNA amplification was also attempted with primer pair HhGF/HhGR (Abel *et al.* 2006), using several different annealing temperatures. Cycling conditions were: initial Hot Start at 95 °C for 15 min, followed by 35 cycles of 94 °C for 40 s, 54–60 °C for 40 s, 72 °C for 45 s; and final extension at 72 °C for 10 min. Genomic DNA from isolates *HtOaVulpes-1982* and *HtAaVulpes-2007* and purified PCR-products from the amplification of isolate *HtRtVulpes-1981* with primers AT264/AT9, were used as templates. Since no PCR products could be obtained with this primer pair, amplification with primer pair AT264/HhGR was attempted instead, using the same cycling conditions as for primer pair AT264/AT9. Genomic DNA from isolates *HtOaVulpes-1982*, *HtAaVulpes-2007* and

*HhCanis-2000*, as well as PCR products from the amplification of these isolates with primer pair AT264/AT9, were used as templates for primer pair AT264/HhGR.

**HSP70 gene.** A portion of the 70 kDa heat shock protein (HSP70) gene was amplified using primer pair HSPF/HSPR (Monteiro *et al.* 2007). Cycling conditions were: initial Hot Start at 95 °C for 15 min, followed by 35 cycles of 94 °C for 40 s, 56 °C for 50 s, 72 °C for 90 s; and final extension at 72 °C for 10 min.

Each PCR reaction mixture contained 3 µl aliquots of the DNA solution, 25 µl of HotStarTaqMaster 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. A negative control was included in each PCR run. All PCR reactions were performed in an iCycler Thermal Cycler (Bio-Rad, USA).

**DNA sequence analysis and GenBank submission.** The PCR products were visualized and purified as previously described (Dahlgren and Gjerde, 2007). Quantity and purity of the PCR products were evaluated on a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, USA). The amplified DNA fragments were sent to Eurofins MWG Operon, Germany, for sequencing on both strands, using the same forward and reverse primers as for the PCR. The application Contig Express in Vector NTI® Advance 10 software (Invitrogen) was used to assemble sequences of each DNA region. The non-redundant nucleotide sequence database, maintained by the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>), was searched for sequences similar to those obtained in this study, using the program BlastN. New DNA sequences from all loci were submitted to GenBank and have been issued Accession numbers GQ984215–GQ984232 (Table 3).

## RESULTS

### *Infection experiments with reindeer-fox isolates of Hammondia triffittae*

Detailed results of the infection experiments are presented in Table 2. Below, only a brief summary of the major findings is given, as well as oocyst measurements. The results of Exps F1–F3, and the major microscopic findings in foxes nos. 39–44 in Exp. F16, have been briefly published before by Gjerde (1983) and Gjerde and Bjerkås (1987), respectively, but have also been included here for the sake of completeness.

**Foxes.** Thirty foxes (21 blue foxes, 8 silver foxes, and 1 silver and blue fox hybrid) shed typical unsporulated *Hammondia* oocysts in their feces.

Table 3. Comparison of new sequences (in bold) obtained from *Hammondia triffittae* oocysts from foxes and *Hammondia heydorni* oocysts from a dog in the present study with other *Hammondia heydorni*-like sequences available in GenBank (referred to by Accession numbers)

DNA region	New sequences of <i>H. triffittae</i> from foxes	Number of nucleotide (nt) positions at which the new sequences of <i>H. triffittae</i> differ from various <i>Hammondia heydorni</i> -like sequences
Ssu rRNA gene	3/3 identical: <b>GQ984222 = GQ984223</b>	0 nt (identical): <b>GQ984224</b> (dog, new) No previous sequences from foxes and dogs in GenBank.
ITS-1	2/2 identical: <b>GQ984215 = GQ984216</b>	0 nt: <b>GQ98417</b> (dog, new) = AF395867 (fox) = DQ997605–10 (dogs) = FJ477299 (crab-eating fox) 1 nt: AF076858 (fox) = AF096501 = DQ997604 ≠ DQ997611 (dogs) 5 nt: <b>GQ984218</b> (dog, new <sup>1</sup> ) = AF317282 = AF508030 = AY189897 = DQ997598–602 (dogs) 6 nt: AF317281 = DQ997603 (dogs)
ITS-2	3/3 identical: <b>GQ984219 = GQ984220</b>	1 nt: <b>GQ984221</b> (dog, new) = AF317281–82 (dogs) No previous sequences from foxes in GenBank.
Lsu rRNA gene	3/3 identical: <b>GQ984225 = GQ984226</b>	0 nt: AF395866 (fox) = AF516885 (fox) 4 nt: <b>GQ984227</b> (dog, new) = AF096502 = AF432125–26 = AF487893 = AY117687 = AY168878 = AF508029 (dogs) 5 nt: AF076870 (dog) 6 nt: AF159240 (dog)
Alpha tubulin gene	3/3 identical: <b>GQ984228 = GQ984229</b>	14 nt: <b>GQ984230</b> (dog, new) = AY169962 = DQ022687 = DQ183059 (dogs) No previous sequences from foxes in GenBank.
Hsp70 gene	2/2 identical: <b>GQ984231 = GQ984232</b>	3 nt: DQ997578 (dog) 7 nt: DQ997585 (dog) 8 nt: DQ997579–82 (dogs) = FJ477298 (crab-eating fox) 9 nt: DQ997583–84 (dogs) 11 nt: DQ997572–75 (dogs) 12 nt: DQ99576–77 (dogs) No sequence obtained from dog in present study; no previous sequences from true foxes in GenBank.

<sup>1</sup> Two different ITS-1 sequences were obtained from 2 subsamples of oocysts from the same dog.

In addition, both blue foxes killed 5 days after infection with goat muscles in Exp. F12, had such oocysts in their colon contents. Foxes shed *Hammondia* oocysts after being fed fresh muscle tissues of either naturally infected reindeer (Exps F1–F3, F7); or experimentally infected lambs (Exps F4, F5, F14), goat kids (Exps F8, F9, F11, F12), blue foxes (Exp. F6) and a rabbit (Exp. F15). In addition, foxes fed tissues of an uninoculated ewe (Exp. F5) and 2 uninoculated goat kids (Exps F10, F12), that had been housed together with inoculated animals, also shed *Hammondia*-type oocysts. The muscle tissues of inoculated intermediate hosts were infective for foxes at least from 16 days post-infection (p.i.) onwards (Goat 1 in Exp. F8). The single fox fed pooled tissues from 3 inoculated cats (Exp. F13), and a fox fed tissues from 1 of 2 inoculated rabbits (Exp. F16), did not shed any oocysts. Neither did the 2 foxes inoculated with oocysts in Exp. F6. None of the animals inoculated with *Hammondia* oocysts showed any clinical signs related to the infections; neither did any of the foxes that were fed infectious muscle tissues and subsequently shed oocysts.

Nine of 10 foxes fed fresh muscle tissues of reindeer in Exps F1–F3 and F7, and 1 fox fed tissues of the 3-year-old ewe in Exp. F5, shed *Sarcocystis*

sporocysts in addition to *Hammondia* oocysts. In contrast to the foxes fed fresh reindeer meat in Exps F1 and F3, none of the 4 foxes fed muscle tissues that had been kept frozen at –20 °C for 2 days or 5 days shed any *Hammondia* oocysts or *Sarcocystis* sporocysts.

The pre-patent period for shedding of *Hammondia* oocysts varied between 3 and 7 days. Most foxes shed oocysts in detectable numbers for about 1 week, but some animals shed oocysts for up to 3 weeks. In most foxes, the onset of oocyst shedding was abrupt, with one or a few days of massive oocyst shedding early in patency, followed by several days with fewer oocysts, or intermittent shedding of oocysts (Table 2).

Unsporulated oocysts were spherical to sub-spherical and had a colourless wall, about 1 µm thick, with no micropyle. The oocysts measured 10.3–15.2 × 9.3–12.8 µm (12.5 ± 0.7 × 10.9 ± 0.5 µm), and their average length to width ratio was 1.15 ± 0.07 (1.00–1.49); *n* = 900. Only 5 of 900 oocysts were measured to be perfectly spherical. There was no significant variation in the average size of oocysts shed by individual foxes on different days during early patency (5–12 days p.i.).

The *Hammondia* oocysts generally sporulated within 3 days at room temperature, even after storage

in a fridge for up to 3 weeks before attempted sporulation. However, oocysts that had been kept frozen at  $-20^{\circ}\text{C}$  for 2 weeks, or oocysts that had been kept at varying temperatures slightly below zero outdoors for 2 weeks, did not sporulate. Sporulated oocysts were spherical to subspherical and measured  $11.9\text{--}13.2 \times 10.8\text{--}12.3 \mu\text{m}$  ( $12.7 \pm 0.4 \times 11.4 \pm 0.4$ );  $n=10$ . The 2 sporocysts within each oocyst were ovoid, thin-walled, and measured  $6.5\text{--}9.5 \times 5.2\text{--}7.2 \mu\text{m}$  ( $8.3 \pm 0.9 \times 6.3 \pm 0.5 \mu\text{m}$ );  $n=40$ . Each sporocyst contained 4 elongate sporozoites.

*Dogs, raccoon dogs, cats, and minks.* None of the 12 dogs that were fed fresh muscle tissues of infected intermediate hosts, shed any *Hammondia* oocysts, but 2 dogs fed fresh muscle tissues of reindeer in Exp. F3, shed *Sarcocystis* sporocysts. The 2 young dogs given fresh muscle tissue in Exp. F1, probably did not ingest any of the infective feed, since they, in contrast to the foxes in that experiment, did not shed any *Sarcocystis* sporocysts either. One of these puppies developed severe diarrhoea and was euthanized 16 days p.i.; the post-mortem examination revealed that the diarrhoea most likely was due to parvo virus infection. None of the 2 raccoon dogs, 3 cats or 4 minks that were fed fresh muscle tissues of inoculated intermediate hosts, shed any *Hammondia* oocysts.

#### *Characteristics of nucleotide sequences obtained in the present study and their similarity to related sequences in GenBank*

The number of DNA-sequences obtained from different loci in the present study, the similarity between them, as well as their GenBank Accession numbers, are listed in Table 3. When all 3 sequences obtained from fox-derived oocysts were identical, only 2 sequences were submitted; usually 1 each from the original reindeer-fox isolate (*HtRtVulpes-1981*) and the moose-fox isolate (*HtAaVulpes-2007*), since isolate *HtOaVulpes-1982* probably was identical with *HtRtVulpes-1981* (obtained after passage of the latter through sheep). The similarity of the new sequences with sequences retrieved from GenBank of related taxa, has also been summarized in Table 3. Below some additional data are provided.

*Ssu rRNA gene.* The complete ssu rRNA gene sequences (1745 bp) obtained from all 3 fox isolates and the dog isolate were identical. These sequences differed at 3 nucleotide positions from *N. caninum* (GenBank nos. AJ271354, U17345 and U17346), at 4 nucleotide positions from *T. gondii* (GenBank no. EF472967), and at 4 nucleotide positions from *H. hammondi* (GenBank no. AF096498). No previous sequences of *H. heydorni* of dogs were available in GenBank for comparison.

*ITS-1.* The complete ITS-1 related sequences (474 bp, including 400 bp of ITS-1) obtained from 2 of the fox isolates (*HtRtVulpes-1982* and *HtAaVulpes-2007*) were identical. No attempt was made to amplify the ITS-1 region of fox isolate *HtRtVulpes-1981*. As for the dog isolate *HhCanis-2000*, 2 subsamples had been subjected to DNA isolation, PCR-amplification and sequencing. The sequences obtained from each subsample differed from each other by 5 nucleotide substitutions. The sequences from the 2 fox-derived isolates were identical with another isolate from a fox, but also identical with 1 of the 2 sequences obtained from our dog isolate, as well as with 7 previous *H. heydorni*-isolates, 6 from dogs and 1 from the crab-eating fox (Table 3). In addition, the 2 fox-derived sequences differed at 1 nucleotide position from another isolate presumably from a fox (GenBank no. AF076858), as well as from 3 sequences of *H. heydorni* from dogs. The second dog-derived sequence, which differed at 5 positions from the fox-derived sequences, was identical with 8 previously sequenced isolates of *H. heydorni* from dogs (Table 3).

*ITS-2.* The complete ITS-2 related sequences (505 bp, including 337 bp of ITS-2) obtained from all 3 fox isolates were identical. No previous fox-derived sequences were available in GenBank for comparison. Our dog-derived sequence was identical with 2 previously sequenced isolates of *H. heydorni* from dogs, and all these sequences differed by a single nucleotide substitution (T/G) from our 3 fox-derived sequences (Table 3). The fox- and dog-derived sequences had only 88% and 87% identities with ITS-2 sequences of *N. caninum* (GenBank no. L49389) and *T. gondii* (e.g., GenBank nos. AJ628253–54), respectively.

*Lsu rRNA gene.* The partial lsu rRNA gene sequences (614 bp) obtained from all 3 fox isolates were identical. They were also identical with 2 previous sequences from foxes (GenBank nos. AF395866 and AF516885). All of these 5 fox-derived sequences differed at the same 4 nucleotide positions from our dog-derived sequence, as well as from 7 previous sequences of *H. heydorni* from dogs. This difference comprised an insert of 2 nucleotides (GG/–) and 2 substitutions (T/C, A/C). Two additional dog-derived sequences in GenBank also differed at these 4 positions, as well as at 1 or 2 other positions (Table 3).

*Alpha tubulin gene.* The partial alpha tubulin gene sequences (278 bp) obtained from all 3 fox isolates were identical. These sequences differed from our dog-derived sequence, as well as from 3 previous GenBank sequences of *H. heydorni* from dogs, at 14 homologous nucleotide positions, comprising 5 nucleotide substitutions and an insert of 9 nucleotides



<i>H. triffittae</i>	G / G / T / G /	ATGCATATGTGTGCATGGAGTGCATGGT / A
<i>H. heydorni</i> a1	C / A / C / T /	ATGCATATGT-----GTGCATGGT / G
<i>H. heydorni</i> a2	C / A / C / T /	ATGCATATGTGTGCATGG-----T / G
Target primer HhGR		ATGCATATGTGTGCATGGAGT

Fig. 2. Nucleotide differences (5 substitutions and an insert) between *H. triffittae* of foxes and *H. heydorni* of dogs in the intron 1 region of the alpha tubulin gene. Due to a repetitive sequence there are 9 possible alignments of the *H. heydorni* sequence at the insert, but only 2 (a1 and a2) are depicted here. When aligned as in a2 it is evident that primer HhGR may also bind fairly well to DNA-sequences of *H. heydorni*, as demonstrated in this study.

in the fox isolates (Fig. 2 and Table 3). No previous fox-derived sequences of this locus were available in GenBank for comparison, but our 3 fox-derived sequences were identical to a 40 nt long fragment of 2 fox-derived sequences, including a 9 nt long insert relative to the dog-derived sequences, depicted in Fig. 3 reported as by Abel *et al.* (2006).

Only a small amount of PCR product was obtained from *Hammondia*-DNA with primers AT264/AT9 under the PCR-conditions used. No PCR products were obtained with primers AT264/AT9 when genomic DNA from either *Sarcocystis alces* or *S. hjorti* was used as template. Primer pair HhGF/HhGR did not amplify DNA from any of the 3 fox-derived *Hammondia* isolates. Primer pair AT264/HhGR, on the other hand, repeatedly yielded PCR products both when genomic DNA from fox-derived (isolates *HtOaVulpes-1982*, *HtAaVulpes-2007*) and dog-derived oocysts (isolate *HhCanis-2000*) were used, and even more so when PCR-products from amplification of the above-mentioned genomic DNA with primers AT264/AT9 were used as templates in the second round of a semi-nested PCR. This was first suggested by similarly sized products on agarose gel, and sequencing of PCR-products from 1 set of such reactions confirmed that amplicons of both *H. triffittae* from foxes and of *H. heydorni* from the dog were generated with primer pair AT264/HhGR.

**HSP70 gene.** The partial HSP70 gene sequences (951 bp) obtained from the 2 fox isolates *HtOaVulpes-1982* and *HtAaVulpes-2007* were identical. No PCR-products could be obtained for this gene from fox isolate *HtRtVulpes-1981*, or from the dog isolate. No previous fox-derived sequences were available in GenBank for comparison, but our fox-derived sequences differed at 3–12 nucleotide positions from 14 previous sequences of *H. heydorni* from dogs, and at 8 positions from a sequence from crab-eating foxes (Table 3). The 3 nucleotide substitutions separating our fox-derived sequences from the most similar sequence of *H. heydorni* in dogs (GenBank no. DQ997578), were also consistently present in all other sequences from dogs and crab-eating foxes. The resulting amino acid sequences, on the other hand, from our fox-derived DNA sequences and the most similar sequence of *H. heydorni* from dogs, were identical.

## DISCUSSION

Comparisons of molecular data obtained in the present study from *Hammondia* oocysts from foxes and a dog respectively, showed that they were genetically different at 4 of 6 DNA loci examined, which suggests that they represent 2 separate, but closely related species, using either foxes or dogs as definitive hosts. Since the dog-derived isolate was genetically consistent with *H. heydorni* of dog origin, characterized by others previously, it follows that the fox-derived isolates were different from this species. Based on our new data, as well as findings reported by others (Schaes *et al.* 2002; Mohammed *et al.* 2003; Abel *et al.* 2006), the *Hammondia* sp. of foxes has been described as a species distinct from *H. heydorni* of dogs, and has been assigned the name *H. triffittae* n. comb. In the following sections, various findings leading to this conclusion will be discussed, and the differences and similarities between *H. triffittae* of foxes and *H. heydorni* of dog-like canids will be outlined.

### *Molecular characteristics of the isolates examined at 6 genetic loci*

The ssu rRNA gene has been extensively used to differentiate between various apicomplexans and to infer their phylogenetic relationships (Šlapeta *et al.* 2003; Morrison *et al.* 2004). However, there are only minor differences between various members of the Toxoplasmatinae (*Toxoplasma gondii*, *Hammondia hammondi*, *Neospora caninum*, *Neospora hughesi*) at this gene (Jenkins *et al.* 1999; Marsh *et al.* 1998; Morrison *et al.* 2004), which was further substantiated in this study by the finding of identical sequences for *H. heydorni* and *H. triffittae*. Similarly, Marsh *et al.* (1998) found no difference at this gene between *N. hughesi* and *N. caninum*, but nevertheless described *N. hughesi* as a new species different from *N. caninum* on the basis of a small difference between their ITS-1 sequences, as well as in some morphological features. Subsequent studies have revealed further differences between these 2 species at other DNA loci, and thus supported their initial proposition (Marsh *et al.* 1999; Walsh *et al.* 2001).

It has been estimated that a common ancestor of the domestic dog and the red fox and arctic



fox existed approximately 10 million years ago (Graphodatsky *et al.* 2008). Based on ssu rRNA gene sequence data, Su *et al.* (2003) have suggested that the divergence of *T. gondii* from *N. caninum* and *H. hammondi* happened about 12 million years ago, and that these taxa diverged on the basis of their choice of definitive hosts. An even more recent split between the dog and fox lineages of *Hammondia* concurrently with the separation of ancient foxes from dogs, might therefore explain the absence of ssu rRNA gene sequence differences between *H. heydorni* and *H. triffittae*.

This study confirmed previous findings that fox-derived ITS-1 sequences might be identical to sequences of *H. heydorni* from dogs (Ellis *et al.* 1999; Schares *et al.* 2002; Monteiro *et al.* 2007) and crab-eating foxes (Soares *et al.* 2009). Hence, *H. triffittae* and *H. heydorni* cannot be differentiated on the basis of this region of the genome. Moreover, the *H. heydorni*-specific primer JS5 designed by Šlapeta *et al.* (2002a) also matches the fox-derived ITS-1 sequences and will therefore presumably also yield a PCR-product for *H. triffittae*.

Two variants of the ITS-1 sequence were obtained from the *H. heydorni* oocysts collected within a few days from the single dog in this study. In contrast, Monteiro *et al.* (2007) examined DNA from *H. heydorni* oocysts derived from 12 dogs at both the ITS-1 and HSP70 gene locus, but found no evidence of mixed infections with multiple variants in individual dogs. Neither have other studies examining a single or a few dog-derived isolates reported such variation within individual isolates (e.g., Ellis *et al.* 1999; Abel *et al.* 2006). It remains to be determined whether such variation in oocyst-derived DNA is due to ingestion of intermediate host tissues infected with 2 separate lineages of *H. heydorni*, or whether individual zoites may harbour different ITS-1 variants in different copies of their rRNA genes. The absence of previous reports about such variation in individual oocyst isolates from either foxes or dogs, favours the first explanation.

In this study, the ITS-2 region of a *Hammondia* sp. from foxes was sequenced for the first time, which revealed a difference at this locus of 1 nucleotide substitution compared with *H. heydorni* from dogs. However, this difference is so small that the ITS-2 region might not be a good marker for differentiating between *H. triffittae* and *H. heydorni*. The D2/D3 domain of the lsu rRNA gene, on the other hand, seems to constitute a more well-suited genetic marker for this purpose, as our results confirmed previous observations (Schares *et al.* 2002; Mohammed *et al.* 2003) that fox- and dog-derived *Hammondia* isolates differ at 4 particular nucleotide positions in this region of the lsu rRNA gene.

The intron 1 region of the alpha tubulin gene also seems to be a good genetic marker for distinguishing between *H. triffittae* and *H. heydorni*. Thus, our

fox-derived *Hammondia* isolates differed at 14 nucleotide positions from all known dog-derived sequences of this gene (present study; Siverajah *et al.* 2003; Abel *et al.* 2006). There were, however, no fox-derived sequences of this gene fragment in GenBank available for comparison, even though Abel *et al.* (2006) apparently sequenced 2 *Hammondia*-isolates from foxes along with 2 *H. heydorni* isolates from dogs. However, in Fig. 3 of their paper (Abel *et al.* 2006), short fragments of both fox- and dog-derived sequences are displayed, but these sequences are actually complementary and reverse to the customary sense strands shown in nucleotide sequence databases. When this is accounted for, it is evident that the 3 fox-derived sequences from our study are identical with their 2 fox-derived sequences in the 40-nt-long region shown, including an insert of 9 nt relative to the dog-derived sequences. Thus, our study confirms the presence of this particular difference between *Hammondia* from foxes and dogs. However, we found an additional difference between fox- and dog-derived *Hammondia*-isolates in this region of the alpha tubulin gene, comprising 5 nucleotide substitutions, which was not mentioned by Abel *et al.* (2006), either because they were absent from their fox isolates, not recognized, or their existence simply not recorded in their paper. Anyway, the 9-nt-long insert/deletion by itself seems to be sufficient to distinguish between *H. triffittae* from foxes and *H. heydorni* from dogs, and the intron 1 region of the alpha tubulin gene therefore seems to constitute a well-suited marker for discrimination between these 2 species.

The small amount of PCR product obtained with primers AT264/AT9 was probably due to the fact that there are only a few copies of the alpha tubulin gene in lower eukaryotes (Siverajah *et al.* 2003). Primer pair HhGF/HhGR, which was designed by Abel *et al.* (2006) to specifically amplify a short sequence of the alpha tubulin gene of *Hammondia* from foxes only, since the reverse primer HhGR allegedly spanned the region of the above-mentioned insert, did not produce any PCR products for any of the fox isolates in this study. As the nucleotide sequence of primer HhGF could not be found within any of the sequenced isolates from either foxes or dogs, or within the 1500 bp long alpha tubulin gene sequence of an isolate of *H. heydorni* available in GenBank (AY169962), it appears that this primer sequence was incorrectly reproduced by Abel *et al.* (2006). Reverse primer HhGR, on the other hand, worked well in combination with forward primer AT264, both with fox- and dog-derived *Hammondia* DNA, even though Abel *et al.* (2006) claimed that primer HhGR was specific for fox-derived *Hammondia* DNA. However, as can be seen from Fig. 2 in this paper, there is only a minor mismatch between primer HhGR and the *H. heydorni* sequence at the very 5'-end of the primer, and this mismatch

was obviously not sufficient to prevent amplification of *H. heydorni*-DNA.

The sequence of the HSP70 coding gene also seems to differ between *H. triffittae* and *H. heydorni*. Thus, our 2 fox-derived sequences differed at 3–12 nucleotide positions from all previously sequenced isolates of *H. heydorni* from dogs (Monteiro *et al.* 2007) and the crab-eating fox (Soares *et al.* 2009), including a consistent difference at 3 particular nucleotide positions. However, due to the apparent sequence variation of *H. heydorni* at the HSP70 gene (Soares *et al.* 2009), and the fact that no other *Hammondia* isolates from foxes so far have been examined at this locus, additional *Hammondia* isolates from both foxes and dogs should be sequenced to determine whether *H. heydorni* and *H. triffittae* are consistently different at this gene, and thus can be unambiguously identified on the basis of this locus.

In summary, the genetic differences that seem to be consistently present between fox- and dog-derived *Hammondia* isolates at the *lsu rRNA* gene and the alpha tubulin gene, and apparently also at the ITS-2 locus and the HSP70 coding gene, strongly suggest that these lineages should be regarded as separate species.

#### Biological characteristics and differences

**Definitive hosts.** The series of infection experiments reported here for the first time (Exps F4–F16), as well as the recently reported infection of foxes with tissues from moose (Dahlgren and Gjerde, 2010), confirmed the initial observations by Gjerde (1983) that the red fox (*Vulpes vulpes*) and the arctic fox (*Vulpes (=Alopex) lagopus*) are equally well suited as definitive hosts for *H. triffittae*. There seem to be no differences between these 2 fox species as regards susceptibility to infection, pattern and timing of oocyst shedding, or the size and shape of the oocysts shed. Similarly, the other *Hammondia* isolates that seem to belong to *H. triffittae* on the basis of their identical *lsu rRNA* gene and partial alpha tubulin gene sequences, have been found in different subspecies of the red fox (Schares *et al.* 2002; Mohammed *et al.* 2003).

In this study, a total of 12 dogs were fed fresh muscle tissues of intermediate hosts (reindeer, sheep, goats) that contained infectious stages of *H. triffittae* as evidenced by the subsequent shedding of *H. triffittae* oocysts by foxes fed concurrently with the dogs. However, the 2 young dogs used in Exp. F1 may not have ingested any muscle tissues, since they, in contrast to the foxes in that experiment, did not shed any *Sarcocystis* sporocysts. Still, at least 10 dogs ingesting infectious stages of *H. triffittae* in muscle tissues of intermediate hosts, did not become infected, whereas nearly all foxes fed such tissues became infected and shed oocysts, as did all 8 foxes

fed muscle tissues of moose in 2007 (Dahlgren and Gjerde, 2010). Consequently, the stages of *H. triffittae* in the muscle tissues of the intermediate hosts seem to be highly infectious for foxes, but non-infectious for dogs. The same seems to be true for the *Hammondia* sp. of foxes in Germany. Thus, Schares *et al.* (2002) could not identify any *Hammondia* oocysts in the faeces of 5 dogs fed in parallel with the 6 foxes that got infected with *Hammondia* sp. Similarly, Entzeroth *et al.* (1978) fed to both a fox and a dog muscle tissues of naturally infected roe deer, but observed *Hammondia*-like oocysts in the faeces of the fox only, whereas both animals shed *Sarcocystis* sporocysts, which also would indicate that this *Hammondia* sp. was infectious for foxes only.

In addition to the apparent specificity of *H. triffittae* for foxes as observed in experimental infections, none of the *H. heydorni*-like oocyst isolates from naturally infected dogs have ever been found to be identical with *H. triffittae* at the D2/D3 domain of the *lsu rRNA* gene or at the alpha tubulin gene. Wapenaar *et al.* (2006) found *Hammondia*-like oocysts in the feces of 2 of 271 free-ranging red foxes in Canada, which were further examined in PCR assays using primers specific for either *H. heydorni* or *N. caninum*. Both isolates were negative for *H. heydorni*, but positive for *N. caninum* with primers Np21/Np6. In a previous study, primers Np21/Np6 did not yield a PCR product for the *HtAaVulpes-2007* isolate of *H. triffittae* (Dahlgren and Gjerde, 2010), and thus it would appear that the Canadian foxes indeed had *N. caninum* oocysts in their feces, but these oocysts might have been ingested with the feed, since foxes experimentally infected with tissue stages of *N. caninum* have not shed any oocysts of this species (Schares *et al.* 2002). The *Hammondia*-like oocysts detected in the feces of 2 of 271 Norwegian red foxes surveyed in 2001 and 2002 (Table 1; Gjerde, unpublished observations), were unfortunately not kept for molecular analysis, but presumably also belonged to *H. triffittae*.

The South American crab-eating fox (*Cerdocyon thous*) has recently been reported to be another definitive host for *H. heydorni*, since an oocyst isolate from this canid had 100% sequence identity at the ITS-1 region and the HSP70 gene with *H. heydorni*-isolates from dogs (Soares *et al.* 2009). However, it is important to note that the crab-eating fox, together with the other South American foxes, form a separate clade within the family Canidae, and that this clade is more distantly related to the clade of red fox-like canids ('true foxes'), which includes the red fox and arctic fox, than to the clade of wolf-like canids, which includes the grey wolf, the dog and the coyote (Bardeleben *et al.* 2005; Graphodatsky *et al.* 2008). It has been estimated that the ancestors of the South American canids and the wolf-like canids diverged from the red fox-like canids about 9–10 million years ago, and that the South American canids and

wolf-like canids diverged about 6–7.4 million years ago (Graphodatsky *et al.* 2008). Thus, it is possible that *H. triffittae* separated from *H. heydorni* when the red fox-like canids separated from the other canids and has since co-evolved with the true foxes (genera *Vulpes* and *Fennecus*). This would imply that South American foxes might be unsuitable as definitive hosts for *H. triffittae*.

The placement of the raccoon dog within the family Canidae is somewhat uncertain, but recent phylogenies either place it within the clade of red fox-like canids, or as a sister taxon to this clade (Bardleben *et al.* 2005; Graphodatsky *et al.* 2008). This would suggest that the raccoon dog might still be a possible definitive host of *H. triffittae* in spite of the seemingly negative outcome of the infection experiment (Exp. F14), which might have been due to examination of too few fecal samples (from day 6 p.i. only).

**Intermediate hosts.** Four separate experiments (Exps F1–F3, F7), of which the first 3 were described by Gjerde (1983), established that reindeer were natural intermediate hosts for *H. triffittae*. Similarly, the experiment in 2007 proved that moose was another natural intermediate host for this species in Norway (Dahlgren and Gjerde, 2010). Exps F4–F6 and F8–F15 further established that sheep, goats, blue (arctic) foxes and a rabbit, but not cats, were experimental intermediate hosts for *H. triffittae*. If we assume that the *Hammondia*-like oocysts shed by foxes fed muscle tissues of various hosts in other studies, also belong to *H. triffittae*, then the guinea pig is another experimental intermediate host (Schaes *et al.* 2002), while sheep (Ashford, 1977; Schaes *et al.* 2002), goats (Schaes *et al.* 2002), roe deer (Entzeroth *et al.* 1978), the Arabian mountain gazelle (Mohammed *et al.* 2003), and possibly also horses (Lee, 1934) may act as additional natural intermediate hosts for this species. As for horses, the foxes and dogs used in Lee's (1934) transmission experiments were apparently fed fresh horse meat, and the shedding of *I. bigemina*-like oocysts by 2 foxes and several dogs inoculated orally with such oocysts in his experiments, was probably the result of ingestion of infective tissue stages contained in the fresh horse meat used as feed, as also suggested by Heydorn (1973). The fact that foxes may also act as intermediate hosts for *H. triffittae*, implies that transmission to foxes may not only occur through predation and scavenging, but also through cannibalism.

The intermediate host range of *H. triffittae* seems to be quite similar to that of *H. heydorni* in that both species use various bovids and cervids for this purpose. The fact that fewer animal species have so far been determined to be intermediate hosts for *H. triffittae* than for *H. heydorni*, is probably mostly due to the fact that relatively few studies have

involved feeding of foxes with fresh muscle tissues of various hosts, compared with the number of such studies conducted with dogs or coyotes. For *H. heydorni*, cattle (Heydorn, 1973; Fayer, 1974), sheep and goats (Dubey and Williams, 1980), water buffalos (Dissanaike and Kan, 1977; Nassar *et al.* 1983), camels (Nassar *et al.* 1983; Warrag and Hussain, 1983), wapiti (Margolin and Jolley, 1979), moose (Dubey and Williams, 1980), and white-tailed deer (Dubey *et al.* 2004) seem to be natural intermediate hosts, of which only sheep, goats and moose so far have been recorded as such for *H. triffittae*. Similar to foxes as regards *H. triffittae*, dogs may act as both definitive and intermediate hosts for *H. heydorni* (Heydorn, 1973; Dubey and Fayer, 1976; Matsui *et al.* 1981), whereas cats do not seem to act either as definitive or intermediate host for any of these species (present study; Heydorn, 1973). From what is presently known, *H. triffittae* might differ from *H. heydorni* in its ability to use rabbits as intermediate hosts. Thus, the single fox fed muscle tissues of an inoculated rabbit in Exp. F15 shed oocysts, whereas none of 5 dogs fed musculature of 3 rabbits inoculated with large numbers of *H. heydorni* oocysts became infected (Heydorn, 1973), and neither did a dog fed tissues of a rabbit that had been inoculated with a moderate number of such oocysts (Matsui *et al.* 1981). However, more experiments are necessary to confirm these findings.

**Development in the definitive host.** The development of *H. triffittae* in the intestine of foxes as reported by Gjerde and Bjerckås (1987) seems to be quite similar to that reported for *H. heydorni* in dogs (Heydorn *et al.* 1975; Dubey and Fayer, 1976). Thus, using light and transmission electron microscopy, Gjerde and Bjerckås (1987) examined the intestinal mucosa of 6 foxes killed 1–5 and 9 days after being fed tissues of an experimentally infected goat (foxes 39–44 and goat Go4 in Exp. F12 in Table 2 in present paper). In the fox killed 5 days p.i., developmental stages were very numerous, and occurred throughout the small intestine, except the duodenum. Schizonts were the predominant stage in the jejunum, while gamonts were numerous in the ileum. All developmental stages in all foxes were almost exclusively localized at the distal end of the villi, mostly at their tips. However, in spite of the large number of developmental stages present, there was only minor damage to the intestinal mucosa (Gjerde and Bjerckås, 1987). The development of oocysts in the posterior portion of the small intestine only, was confirmed in the 2 *Hammondia*-infected foxes killed 7 days after being fed tissues of naturally infected moose (Dahlgren and Gjerde, 2010). In that study *Hammondia* oocysts were only seen in mucosal scrapings from the posterior half of the small intestine, whereas *Sarcocystis* oocysts occurred throughout the entire small intestine. Moreover, the pre-patent and patent



period, pattern of oocyst shedding, and oocyst morphology are also closely similar between *H. truffittae* and *H. heydorni*. Hence, these species cannot be distinguished on the basis of oocyst morphology, and molecular methods using appropriate genetic markers are necessary to accomplish this, as demonstrated in this study.

*Development in the intermediate host.* So far, there is only scant knowledge about the development of *H. truffittae* in its intermediate hosts, and about the nature of its infectious tissue stage. However, many haematoxylin and eosin-stained histological sections were examined from various muscles of the 2 lambs (Sh1 and Sh2) inoculated with oocysts and fed to foxes in Exps. F4 and F5. Only 7 cyst-like structures were found within striated muscle cells, each measuring 10–20 µm in greatest diameter and containing a small number of zoites (Gjerde, unpublished observations). Whether these 'cysts' had been derived from the inoculation of the lambs with oocysts of *H. truffittae*, or from other infections, e.g., *T. gondii*, could not be conclusively determined. Nevertheless, these small cysts are consistent with 'cysts' of *Hammondia* sp. of foxes observed in tissue culture by Schares *et al.* (2003). Similarly, *H. heydorni* of dogs also seems to form very small tissue cysts. Thus, Matsui (1991) found only 2 small cysts, about 10–12 µm in diameter, in the brain of 2 guinea pigs killed 77 and 189 days, respectively, after infection with a high number of oocysts from dogs.

In the present study, infectious stages of *H. truffittae* seemed to be present in the muscle tissues of intermediate hosts at least by 16–20 days p.i., since the ingestion of such tissues by foxes resulted in oocyst shedding. This is in agreement with the *in vitro* development of *Hammondia* sp. of foxes reported by Schares *et al.* (2003), who found that all parasitophorous vacuoles present in culture cells 19 days p.i. seemed to represent cyst stages. Similarly, for *H. heydorni* from dogs, Matsui *et al.* (1981) found that tissues of inoculated guinea pigs and dogs had become infectious for dogs by 20 days p.i., but not by 10 days p.i., whereas Matsui (1991) found that tissues of infected guinea pigs were infectious for dogs 21 days p.i. and later, but not on days 5, 9 or 14 after oocyst inoculation. Thus, *H. truffittae* and *H. heydorni* seem to develop in a closely similar manner in their intermediate hosts.

#### *Relationship between H. truffittae and previous Hammondia-like oocyst isolates from foxes*

It is reasonable to believe that the *Isospora bigemina*- or *Hammondia*-like oocysts found in foxes in various other studies summarized in Table 1, also belong to *H. truffittae*, although indistinguishable oocysts of other species might occasionally occur as spurious

parasites, as suggested by the possible finding of *N. caninum* oocysts in 2 foxes by Wapenaar *et al.* (2006). In particular, the size and shape of the oocysts originally designated *Isospora truffitti* by Nukerbaeva and Svanbaev (1973) are fully consistent with the oocysts of *H. truffittae* as described in the present paper. The same is true for the oocysts found in foxes in Belarus and designated *I. truffitti* by Gerasimchik (2005). Moreover, no studies have so far indicated that foxes may be the natural definitive hosts of a valid *Isospora* species with oocysts indistinguishable from those of *H. truffittae*; all true *Isospora* species of foxes having distinctly larger oocysts (Nukerbaeva and Svanbaev, 1973; Golemanski and Ridzhakov, 1975).

No molecular data accompanied the original description of *Isospora truffitti* by Nukerbaeva and Svanbaev (1973), and those oocysts are probably no longer available for molecular studies. Similarly, no molecular data were presented by Gerasimchik (2005, 2006) when the same species name was applied to some of the oocysts identified in foxes in Belarus. Hence, the exact relationship of these oocysts to the presently examined *Hammondia* sp. of foxes will probably never be revealed. However, in order to avoid any further confusion, the amended species designation (Levine, 1985) has been retained for the *Hammondia* sp. of foxes described in the present paper, but the former species *I. truffitti*/*I. truffittae* has been transferred to the genus *Hammondia* (family: Sarcocystidae) because of its heteroxenous life cycle and close relationship to *Hammondia heydorni* of dogs.

#### TAXONOMIC SUMMARY

*Name:* *Hammondia truffittae* (syn. *Isospora truffitti*, *I. truffittae*)

*Description:* Oocysts (Figs 1 B–F) spherical to subspherical, with wall about 1 µm thick, colourless; micropyle and oocyst residuum absent; unsporulated oocysts 12.5 ± 0.7 × 10.9 ± 0.5 µm (10.3–15.2 × 9.3–12.8 µm, n = 900), with L:W ratio 1.15 ± 0.07 (1.00–1.49, n = 900); sporulated oocysts 12.7 ± 0.4 × 11.4 ± 0.4 µm (11.9–13.2 × 10.8–12.3 µm, n = 10); two sporocysts, each with 4 sporozoites; sporocysts ovoid 8.3 ± 0.9 × 6.3 ± 0.5 µm (6.5–9.5 × 5.2–7.2 µm, n = 40); sporozoites elongate.

*Type definitive hosts:* The red fox (*Vulpes vulpes*) and the arctic fox (*Vulpes lagopus*). Life cycle obligatory heteroxenous.

*Type intermediate hosts:* Reindeer (*Rangifer tarandus*) and moose (*Alces alces*).

*Other intermediate hosts:* Sheep, goats, rabbits and arctic foxes (experimental infections).

*Type locality:* Norway.

*Site of infection in definitive hosts:* Schizogony mainly in jejunum; gamogony mainly in posterior



jejunum and ileum. Development mainly at tips of intestinal villi (Gjerde and Bjerås, 1987).

*Pre-patent period:* 3–7 days, usually 6–7 days.

*Patent period:* About 3 weeks; high oocyst excretion usually only during first week of patency.

*Sporulation time:* About 3 days at room temperature (22–24 °C).

*Site of infection in intermediate hosts:* Not conclusively determined, but probably very small, thin-walled cysts (10–20 µm in diameter) formed in striated muscle cells; each cyst containing only a few zoites.

*Material deposited:* Oocysts and phototypes of oocysts of *H. triffittae* from foxes of isolates *HtRt-Vulpes-1981* (reindeer/fox) and *HtAaVulpes-2007* (moose/fox), as well as oocysts of *H. heydorni* from a dog (isolate *HhCanis-2000*) have been deposited at the National History Museum, Oslo, Norway, and have been issued collection numbers NHMO-Prot00012, NHMO-Prot00013 and NHMO-Prot00014, respectively. Nucleotide sequences of *H. triffittae* from 6 genetic markers have been submitted to GenBank, and have been issued Accession numbers GQ984215–16 (ITS-1), GQ98422–23 (ssu rRNA gene), GQ984219–20 (ITS-2), GQ944225–26 (lsu rRNA gene), GQ944228–29 (alpha tubulin gene), and GQ944231–32 (HSP70 gene).

*Etymology:* Nukerbaeva and Svanbaev (1973) named an *Isospora* species of foxes *Isospora triffitti* in recognition of parasitologist Marjorie J. Triffitt, who first described similar oocysts from foxes. Levine (1985) amended this name to *I. triffittae*, since M. J. Triffitt was a woman.

*Remarks:* The oocysts of *Hammondia triffittae* in foxes are morphologically indistinguishable from oocysts of *Hammondia heydorni* in dogs and other wolf-like canids, and can only be unambiguously differentiated from the latter by molecular methods using the appropriate genetic markers. However, true foxes do not seem to act as definitive hosts for *H. heydorni*, and neither do dogs for *H. triffittae*.

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