What is *Cryptosporidium*? Reappraising its biology and phylogenetic affinities

John R. Barta[1] and R.C. Andrew Thompson[2]

[1] Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ont, N1G 2W1, Canada
[2] World Health Organization Collaborating Centre for the Molecular Epidemiology of Parasitic Infections, School of Veterinary and Biomedical Sciences, Murdoch University, WA 6150, Australia

In raising the question 'What is *Cryptosporidium*?', we aim to emphasize a growing need to re-evaluate the affinities of *Cryptosporidium* species within the phylum Apicomplexa so as to better understand the biology and ecology of these parasites. Here, we have compiled evidence from a variety of molecular and biological studies to build a convincing case for distancing *Cryptosporidium* species from the coccidia conceptually, biologically and taxonomically. We suggest that *Cryptosporidium* species must no longer be considered unusual or unique coccidia but rather seen for what they are – a distantly related lineage of apicomplexan parasites that are not in fact coccidia but that do occupy many of the same ecological niches. Looking at *Cryptosporidium* species without traditional coccidian blinders is likely to reveal new avenues of investigation into pathogenesis, epidemiology, treatment and control of these ubiquitous pathogens.

A crack appears...

Although believed for many years to be coccidia (see Glossary) [1], *Cryptosporidium* species were always viewed as atypical in light of their unusual autoinfective oocyst, their strange association with their host cell and their complete insensitivity to anticoccidial drugs (reviewed in Ref. [2]) (Table 1). An unexpected report of serological cross-reactivity with a *Monocystis* sp. gregarine [3] widened the crack of disbelief in this relationship with coccidia, and the crack became a fissure when molecular tools provided convincing evidence that *Cryptosporidium* species share a common ancestor with what were thought to be distantly related apicomplexan protists, the gregarines, rather than with the coccidia [4]. Put a different way, the coccidia have now been recognized to be more closely related to malaria and haemogregarine blood parasites than they are to *Cryptosporidium* species, despite the latter being traditionally considered 'coccidia'.

Taxonomy of *Cryptosporidium*: from mice to men

Although described in the early 1900s by Tyzzer from mice [5], *Cryptosporidium species* did not become a major focus of research until the mid 1970s, when the first human cases

of cryptosporidiosis were reported, closely followed by the emergence of *Cryptosporidium* as a life threatening opportunistic infection in AIDS patients [6]. The increased scrutiny given to the parasite resulted in a period of taxonomic confusion concerning the status of morphologically similar *Cryptosporidium* 'species' occurring in a variety of hosts [7]. Although rationalization initially won the day in the late 1980s, the advent of molecular tools for parasite characterization, driven mainly by the demands of the water industry to identify sources of contamination,

> ### Glossary
>
> **Apicoplast**: recently discovered plastid (genome-containing) organelle in many members of the Apicomplexa homologous to chloroplasts and considered to be of secondary endosymbiotic origin.
> **Archigregarines**: gregarine parasites of the intestines of marine invertebrates exhibiting three types of multiplication (merogony, gametogony and sporogony); considered to be among the earliest diverging apicomplexan parasites.
> **Eugregarines**: gregarine parasites of the intestine and other organs of marine and terrestrial invertebrates exhibiting only sporogony and gametogony; considered to be derived from the archigregarines.
> **Coccidia**: intracellular parasites of the gut and other organs of vertebrates, with alternating asexual and sexual developmental phases of development resulting in the production of an environmentally resistant oocyst (usually in the feces of the definitive host); species are usually host specific.
> **Epimerite**: anterior part of gregarine cell containing the apical complex, usually involved in attachment to host.
> **Gamont**: sexual stage in the sporozoan life cycle, produced by gametogony from a trophozoite or a merozoite (syn gametocyte); a pre-gamete.
> **Gregarines**: extra- and/or intracellular protozoan parasites with large mature gamonts that usually develop extracellularly with most exhibiting syzygy in their developmental cycles.
> **Haemogregarine**: apicomplexan intracellular adeleorinid parasites that parasitize the blood of vertebrates and are transmitted by a wide variety of invertebrate definitive hosts.
> **Merogony**: asexual reproduction, commonly by multiple fission of the parasite nucleus followed by simultaneous cellular division producing daughter cells (merozoites); asexual schizogony.
> **Meront**: asexual multinucleate stage that forms during merogony (an asexual schizont).
> **Monoxenous**: complete parasite development requiring only one individual.
> **Myzocytosis**: predatory mode of feeding in which parasite cell pierces the cell wall and/or membrane of the prey (host) cell with a feeding tube, and sucks out the cellular content and digests it.
> **Oocyst**: first structure formed immediately following syngamy (fusion of gametes) in apicomplexan protists; contains sporozoites.
> **Pairing**: see syzygy.
> **Sporozoite**: first haploid motile stage of apicomplexan parasites formed after syngamy (fusion of gametes); found in oocysts.
> **Syzygy**: association of gamonts (pre-gametes) end to end or in lateral pairing prior to the formation of gametes, found in most gregarine protists and, perhaps, piroplasms.
> **Trophozoite**: active, non-encysted, feeding and/or resting stage of a protozoan parasite.

Corresponding author: Thompson, R.C.A. (a.thompson@murdoch.edu.au).
Available online 14 August 2006.

Table 1. Differences between *Cryptosporidium* and the Coccidia[a]

Property	*Cryptosporidium*	Coccidia
Location within the host cell	Intracellular but extracytoplasmic	Completely intracellular
Attachment or feeder organelle	Present	Not present
Morpho-functional types of oocyst	Two types: thick and thin-shelled	Only thick-shelled
Size of oocysts	Small ($5–7.4 \times 4.5–5.6$ μm)	Larger ($9–38 \times 7–39$ μm)
Sporocyst, micropyle and polar granules in oocyst	Lacking	Present
Extracellular development	Yes	No
Syzygy-like pairing of extracellular stages	Yes	No
Apicoplast	Lost	Present
Complexity of biosynthetic pathways	Simplified; reliant on salvaging from host	More complex (where studied)
Sensitivity to anticoccidial drugs	Insensitive	Sensitive
Host specificity	Low	High
Pathogenesis	Not understood	Mainly understood

[a]A major difference is that molecular phylogenetic studies group *Cryptosporidium* as a clade separate from coccidian taxa.

has led to a proliferation of re-described and newly described species. Most of these species appear to be mainly host specific [8].

Molecular phylogeny

The development of molecular tools for the genetic characterization of *Cryptosporidium* species also provided the opportunity for phylogenetic studies. These confirmed that *Cryptosporidium* were not as closely related to coccidian parasites as originally suspected [9], but rather were closer to the gregarines [4,10–13]. Thus, within the digestive tract of vertebrates, there are at least two distinct lineages of apicomplexan parasites that have exploited this ecological niche [10–12] (Figure 1). The first lineage is the classically recognized coccidian, including eimeriid (Eimeriidae) and isosporoid (Sarcocystidae) coccidian, which are each well supported natural groups of taxa that share a common ancestor to the exclusion of all other apicomplexan parasites studied to date. The second lineage appears to be restricted to only *Cryptosporidium* species, which have arisen early in the diversification of ancestral apicomplexan parasites and share a most recent common ancestor with gregarines. Perhaps the most interesting aspect of a link between *Cryptosporidium* and the gregarines suggested by phylogenies derived from small subunit ribosomal DNA sequences is that cryptosporidian parasites might be most closely related to some of the earliest diverging apicomplexan parasites, the archigregarines [12]. In addition to molecular evidence, similarities between the two groups include a monoxenous life cycle, oocysts with four sporozoites, a usual location in the host gastrointestinal tract and extracellular gamonts or trophozoites [14]. However, it is perhaps comparing the feeding behaviours of archigregarines with that of *Cryptosporidium* that is most intriguing.

The host–parasite interface

Cryptosporidium species are confined to the apical surfaces of epithelial cells, principally in the intestine. Although development has been considered intracellular, *Cryptosporidium* characteristically take up a unique extra-cytoplasmic or epicellular location within host cells. *Cryptosporidium* parasites attach to the apical surfaces of host cells and, by a process that is not understood, become internalized within an extracytoplasmic compartment, overlaid by the host cell membrane but separated

from the host cell cytoplasm by an electron dense layer that appears to be of host origin (reviewed in Ref. [2]). A tunnel directly connecting the parasite to the host cell forms during internalization [15]. The formation of this tunnel seems to be a first step in the subsequent development of
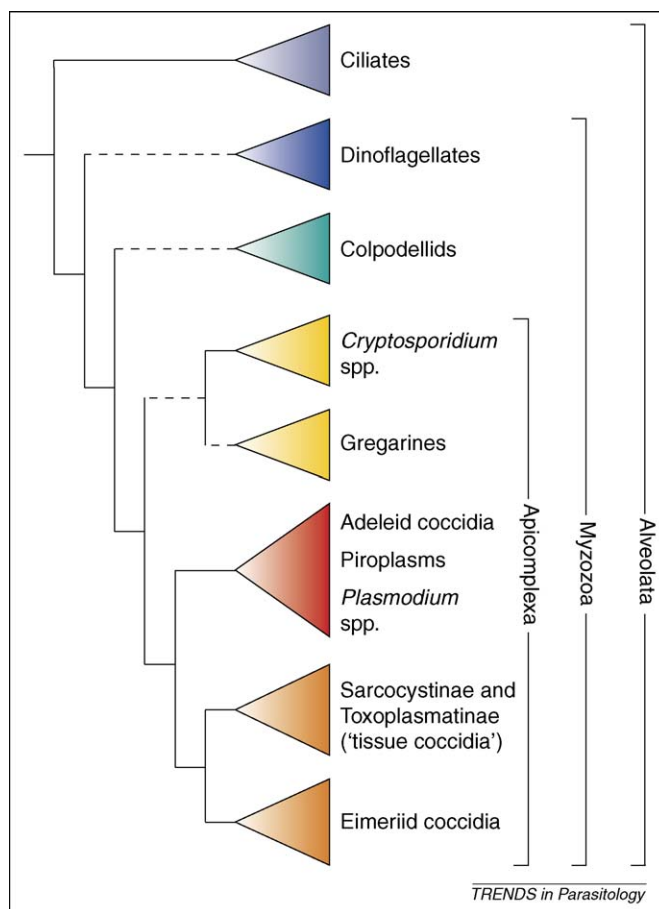

Figure 1. Representation of the probable evolutionary relationships among major groups within the Alveolata, based principally on 18S small subunit ribosomal RNA sequences. Only the branching order among the taxa is shown. Uncertainty regarding the monophyly of several of these widely recognized groups is represented as dotted horizontal lines. The monophyly of the Apicomplexa is well supported, with many of the traditional groups of apicomplexan parasites, such as the malarial parasites or tissue coccidia, shown to be natural (monophyletic) groupings. By contrast, the relationships of *Cryptosporidium* species to other members of the phylum Myzozoa [13] within the Alveolata is considerably different from its historically assumed position within the eimeriid coccidian: *Cryptosporidium* species probably arose from a common alveolate ancestor shared with gregarines that is only distantly related to the coccidia. Given these relationships, it is not surprising that *Cryptosporidium* species do not behave like coccidia, as there is a large evolutionary distance between these two groups of apicomplexan parasites that both infect the epithelium of vertebrates.
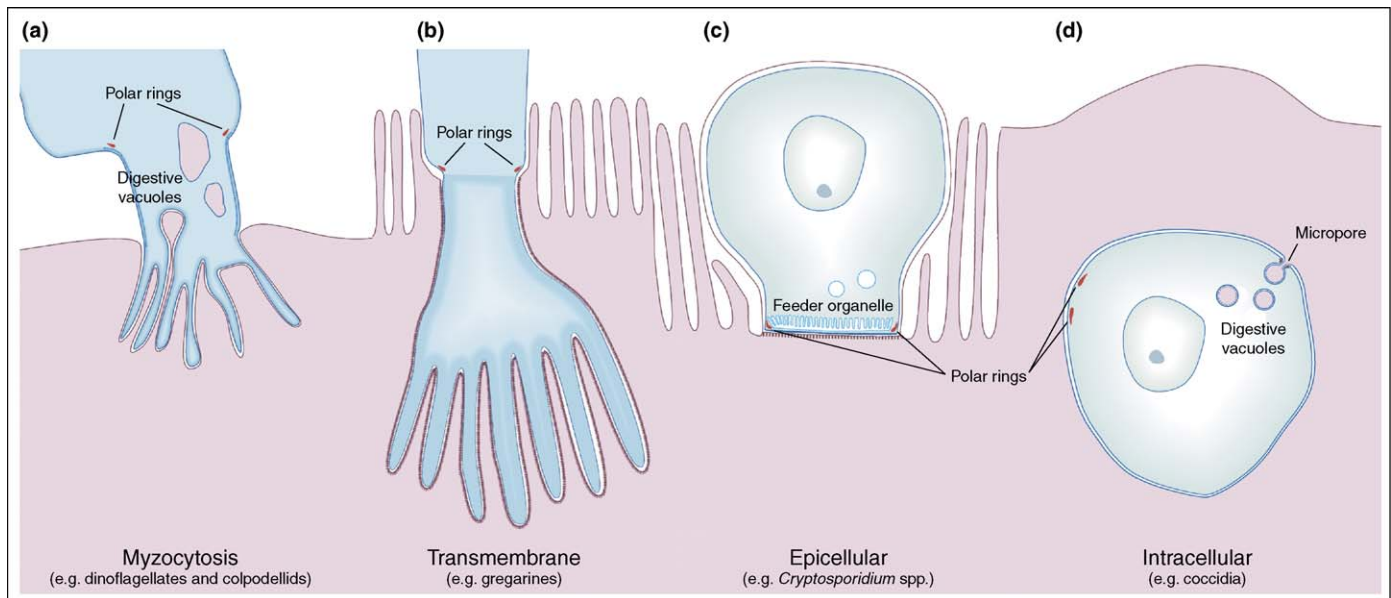
Figure 2. Host–parasite interactions in **(a)** dinoflagellates, **(b)** gregarines, **(c)** *Cryptosporidium* species and **(d)** coccidia. The micropredation exhibited by dinoflagellates, perkinsids and colpodellids has been termed 'myzocytosis' or cellular vampirism. Myzocytotic feeding by dinoflagellates on both protistan prey and vertebrate epithelial cells has been demonstrated. Within the Apicomplexa, the gregarines infecting marine and terrestrial invertebrates have apparently elaborated this host association into a more permanent attachment, sometimes with partial or complete intracellular localization. Ancestral myzocytotic feeding in *Cryptosporidium* species has evolved into an epicellular association with the vertebrate epithelial cell characterized by an elaborate membranous feeding organelle that develops from the apical region of the zoite after internalization of the parasite within the host cell (the remnant of the polar ring complex is indicated in red). We propose that the intimate host association of the epimerite of gregarine trophozoites and the development of the feeding organelle by trophozoites of *Cryptosporidium* species is derived evolutionarily from the micropredatory feeding methods of their shared common myzozoic ancestor. Nutrients are apparently taken up through transmembrane transport in the apical region in *Cryptosporidium* spp. In other intracellular Apicomplexa, both transmembrane transport of necessary molecules and ingestion of host cell cytoplasm through active micropores (located in the plasmalemma of trophozoites in regions other than the apical complex) are used by various parasites. Diagrams are not to scale.

the unique, yet ill-defined, multi-membranous feeder organelle of *Cryptosporidium* species that is presumably involved in the uptake of nutrients from the host cell [2]. The similarities between this method of feeding and that seen in many gregarines are striking (Figure 2).

The earliest stages of apicomplexan evolution involved the development of intracellular invasion of animal cells with a transition from myzocytosis [16]. This is seen in some archigregarines, such as *Selenidium*, a parasite of marine polychaetes that uses myzocytosis when feeding on cells of the host gut lining [17,18]. Myzocytosis has been described as a form of 'cellular vampirism' in which the predatory cell pierces the cell wall and/or membrane of the prey cell with a feeding tube, sucks out the cellular contents and digests them [12]. Examination of early alveolate evolution suggests that dinoflagellates and primitive apicomplexan parasites use myzocytosis in their micropredatory and parasitic roles, respectively [12,14]. This suggests that the feeding organelle that is seen in *Cryptosporidium* species is an evolutionary modification to the ancestral myzocytotic morphological adaptations. Apart from size, the only difference between the two modes of feeding is that *Cryptosporidium* has evolved a way to induce the host cell to overlay it with an extension of the host cell membrane and physical ingestion of host cell cytoplasm has not been observed, as it has for dinoflagellates, perkinsids, colpodellids and some gregarines.

From genome to phenome
Analysis of the complete genome sequences of *Cryptosporidium parvum* and *Cryptosporidium hominis* [19,20] has not only served to reinforce the uniqueness of *Cryptosporidium* compared with other apicomplexan

parasites, but has also revealed its elegant adaptation to parasitism. Its novel metabolic activities are functionally streamlined with a loss and/or simplification of conventional biosynthetic pathways, with the result that *Cryptosporidium* is reliant on maximizing the number of biosynthetic molecules that it can salvage from the host [2,19,20]. *Cryptosporidium* species have anaerobic and aerobic pathways, giving them environmental flexibility, and, perhaps unexpectedly for an enteric apicomplexan, they do not have variant surface proteins. Predictably, analysis of the genome sequences also revealed the absence of conventional drug targets.

Phylogenomic approaches have also helped to resolve some questions about the apparent absence of the apicoplast in *Cryptosporidium*. It would appear that the ancestor to extant *Cryptosporidium* species lost its morphologically identifiable plastid during evolution but that the parasite has retained functional genes associated with the apicoplast in its nuclear genome [21]. It is not known whether any gregarines retain a morphologically distinct apicoplast, but a recent ultrastructural study of the eugregarine *Leidyana* failed to find one [22]. Cavalier-Smith [23] suggested that multiple plastid losses and replacements could have occurred in the alveolates and related eukaryotes but that the ancestor to all alveolates was derived from a plastid-bearing eukaryote. Thus, even if visible plastids are no longer present, some genetic evidence of their existence probably persists in gregarines, as has been demonstrated for *Cryptosporidium* species.

Revelations from *in vitro* culture
The non-coccidian, gregarine affinities of *Cryptosporidium* are further supported by recent developmental studies.
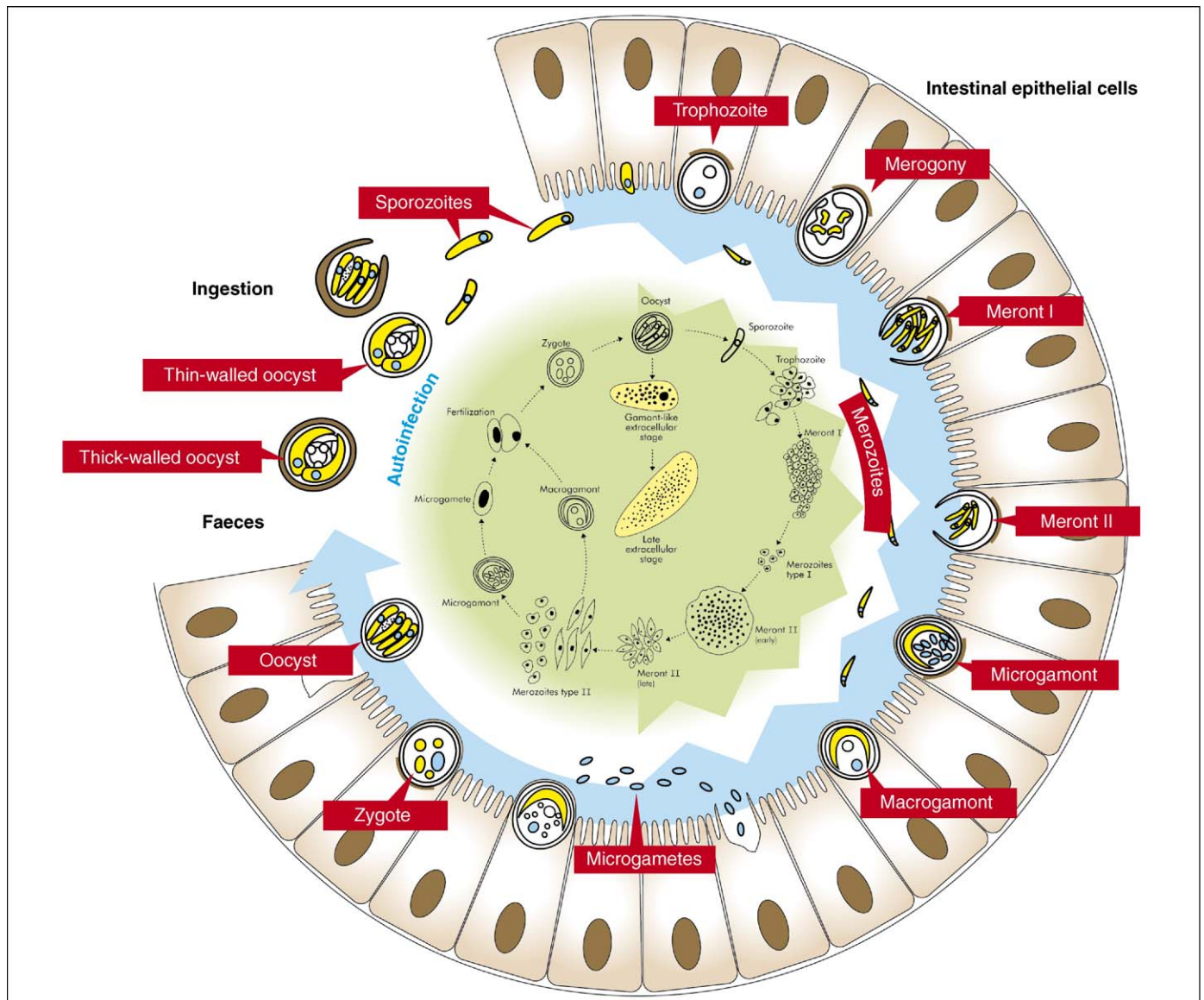
Figure 3. The life cycle of *Cryptosporidium* spp. in the intestine, showing what is known concerning 'intracellular' and extracellular phases. The interaction between the two phases has still to be determined but novel gamont stages appear to develop only as extracellular stages. Parts of figure redrawn from Ref. [26].

The ability to observe the life cycle and development of *Cryptosporidium in vitro*, in both cell-associated and cell-free culture, has demonstrated the occurrence of previously unrecognized stages in the life cycle (Figure 3), particularly trophozoites and extracellular gamont-like stages that grow and develop outside the host cells ([24–27]; L. Zhang *et al.*, personal communication). Similar stages have also been purified from mice infected with *C. parvum* [25]. Stages of *Cryptosporidium* observed in cell-free culture show remarkable similarities to those seen in the life cycles of some gregarines [25,26]. *Cryptosporidium* sporozoites, once they are released from oocysts transform into trophozoites that aggregate, leading to two merogony stages with merozoites from meront II initiating the sexual stage in the life cycle. This behaviour is similar to the developmental stages occurring in the life cycle of the gregarine *Mattesia dispora* [28]. *Cryptosporidium* also has an unusual developmental plasticity in its life cycle, with the ability

to avoid merogony and initiate mitotic division from fused sporozoites [26]. Recent observations *in vitro* have also demonstrated pairing between different developmental stages of *Cryptosporidium* that resembles previously described syzygy in gregarines [26,27]. Pairing of stages of unequal ages and early in development was frequently observed in cell culture and cell-free culture of *Cryptosporidium*. Apart from macro- and microgamonts, pairing was observed among sporozoites, trophozoites and merozoites of *Cryptosporidium* [26].

The occurrence of predominantly extracellular stages in the life cycle that can be completed in cell-free culture has demonstrated that *Cryptosporidium* is not as obligately intracellular as previously thought. Perhaps predictably, the greatly reduced biochemical repertoire retained by *Cryptosporidium* species after loss of both a functional mitochondrion and an apicoplast requires a nutrient-rich environment from which to salvage its metabolic needs.

Conclusions and future perspectives

There is overwhelming evidence that *Cryptosporidium* should be placed in a taxonomic grouping separate from that of the coccidia and closer to that of the gregarines. The final taxonomic placement among or as a sister group to the widely varied parasites collectively referred to as gregarines requires much additional study, including determination of the nearest extant relative of *Cryptosporidium* species. We hope that this article will stimulate such work and ultimately result in a consensus being reached in the future. Formalizing such a shift in taxonomy will properly move attention away from how *Cryptosporidium* species behave differently than 'other coccidia' but focus it more properly on the distinct biological characteristics of *Cryptosporidium* species and its closest living relatives, the gregarines. The understanding of a shared ancestry for cryptosporidian parasites with gregarines will have a significant impact on how we understand and deal with the basic biology of cryptosporidian parasites [13,29], and on how we view the 'primitive' gregarines found in many invertebrates. It might be possible to exploit this relationship at a practical level in the future, by using gregarine parasites as more accessible laboratory models for cryptosporidiosis, particularly for drug discovery. Are there *Cryptosporidium* species waiting to be discovered (or biologically similar apicomplexan parasites, perhaps an archigregarine) that infect the alimentary tract of invertebrates?

Examining the similarities among cryptosporidian and gregarine parasites highlights the paucity of knowledge regarding gregarines among many other apicomplexan parasites of little direct veterinary or medical interest. In particular, the evolution of feeding mechanisms used within the Myzozoa (dinoflagellates and apicomplexan parasites) has not been studied ultrastructurally in anywhere near sufficient detail. Such studies will not only provide clues about the evolution of intracellular parasitism, but in the case of *Cryptosporidium* will also provide a better understanding of the host–parasite relationship. How does *Cryptosporidium* create its own intracellular niche just beneath the host plasma membrane [30]?

We also know little about the pathogenesis of cryptosporidial infections and the relative contribution of the 'intracellular' and extracellular phases of development to disease processes and epidemiology. Dionisio [31] has noted that no correlation has been found between the histological intensity of *Cryptosporidium* infections and clinical severity [32]. Furthermore, the realization that the life cycle of *Cryptosporidium* includes novel developmental stages similar to those of gregarines will contribute to a greater understanding of the environmental ecology of *Cryptosporidium*, which is fundamental to its control. Advances in *in vitro* cultivation, proteomics and the availability of complete genome sequences for the two most important *Cryptosporidium* species of public-health and veterinary significance will provide an excellent resource for 'rediscovering' *Cryptosporidium*.

References

1 Levine, N.D. (1988) *The Protozoan Phylum Apicomplexa* (Vols 1 and 2), CRC

2 Thompson, R.C.A. *et al.* (2005) *Cryptosporidium* and cryptosporidiosis. *Adv. Parasitol.* 59, 77–157

3 Bull, S. *et al.* (1998) Cross-reaction of an anti-*Cryptosporidium* monoclonal antibody with sporocysts of *Monocystis* species. *Vet. Parasitol.* 77, 195–197

4 Carreno, R.A. *et al.* (1999) *Cryptosporidium* is more closely related to the gregarines than to coccidia as shown by phylogenetic analysis of apicomplexan parasites inferred using small-subunit ribosomal RNA gene sequences. *Parasitol. Res.* 85, 899–904

5 Tyzzer, E.E. (1912) *Cryptosporidium parvum* (sp. nov.), a coccidian found in the small intestine of the common mouse. *Archiv. Fur Protistenkund.* 26, 394–418

6 Hunter, P.R. and Thompson, R.C.A. (2005) The zoonotic transmission of *Giardia* and *Cryptosporidium*. *Int. J. Parasitol.* 35, 1181–1190

7 O'Donoghue, P.J. (1995) *Cryptosporidium* and Cryptosporidiosis in man and animals. *Int. J. Parasitol.* 25, 139–195

8 Cacciò, S.M. *et al.* (2005) Unravelling *Cryptosporidium* and *Giardia* epidemiology. *Trends Parasitol.* 21, 430–437

9 Morrison, D.A. and Ellis, J.T. (1997) Effects of nucleotide sequence alignment on phylogeny estimation: a case study of 18 rDNA of Apicomplexa. *Mol. Biol. Evol.* 14, 428–441

10 Leander, B.S. *et al.* (2003) Molecular phylogeny and surface morphology of marine aseptate gregarines (Apicomplexa): *Selenidium* spp. and *Lecudina* spp. *J. Parasitol.* 89, 1191–1205

11 Leander, B.S. *et al.* (2003) Phylogeny of gregarines (Apicomplexa) as inferred from small-subunit rDNA and β-tubulin. *Int. J. Syst. Evol. Microbiol.* 53, 345–354

12 Leander, B.S. and Keeling, P.J. (2004) Morphostasis in alveolate evolution. *Trends Ecol. Evol.* 18, 395–402

13 Cavalier-Smith, T. and Chao, E.E. (2004) Protalveolate phylogeny and systematics and the origins of sporozoa and dinoflagellates (Phylum Myzozoa nom. nov.). *Eur. J. Protistol.* 40, 185–212

14 Leander, B.S. and Keeling, P.J. (2004) Early evolutionary history of dinoflagellates and apicomplexans (Alveolata) as inferred from HSP90 and actin phylogenies. *J. Phycol.* 40, 341–350

15 Huang, B.Q. *et al.* (2004) *Cryptosporidium parvum* attachment to and internalization by human biliary epithelia *in vitro*: a morphologic study. *J. Parasitol.* 90, 212–221

16 Schnepf, E. and Deichgraber, G. (1984) "Myzocytosis", a kind of endocytosis with implications to compartmentalization in endosymbiosis: observations on *Paulsenella* (Dinophyta). *Naturwissenschaften* 71, 218–219

17 Schrevel, J. (1968) L'ultrastructure de la region anterieure de la gregarine *Selenidium* et son interet pour l'etude de la nutrition chez les sporozoaires. *J. Microscop.* 7, 391–410

18 Schrevel, J. (1971) Observations biologique et ultrastructurales sur les Selenidiidae et leurs consequences sur la systematique des gregarinomorphes. *J. Protozool.* 18, 448–470

19 Abrahamsen, M.S. *et al.* (2004) Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. *Science* 304, 441–445

20 Xu, P. *et al.* (2004) The genome of *Cryptosporidium hominis*. *Nature* 431, 1107–1112

21 Huang, J. *et al.* (2004) *Cryptosporidium parvum:* phylogenomic evidence for organelle loss, intracellular and horizontal gene transfer. *Genome Biol.* 5, R88

22 Valigurova, A. and Koudela, B. (2005) Fine structure of trophozoites of the gregarine *Leidyana ephestiae* (Apicomlexa: Eugregarinida) parasitic in *Ephestia kuehniella* larvae (Lepidptera). *Eur. J. Protis.* 41, 209–218

23 Cavalier-Smith, T. (2002) The phagotrophic origin of eukaryotes and phylogenetic classification of Protozoa. *Int. J. Syst. Evol. Microbiol.* 52, 297–354

24 Hijjawi, N.S. *et al.* (2001) Complete development and long-term maintenance of *Cryptosporidium parvum* human and cattle genotypes in cell culture. *Int. J. Parasitol.* 31, 1048–1055

25 Hijjawi, N.S. *et al.* (2002) Successful *in vitro* cultivation of *Cryptosporidium andersoni* with evidence for the existence of novel extracellular stages in the *Cryptosporidium* life cycle. *Int. J. Parasitol.* 32, 1719–1726

26 Hijjawi, N.S. *et al.* (2004) Complete development of *Cryptosporidium parvum* in host cell-free culture. *Int. J. Parasitol.* 34, 769–777

27 Rosales, M.J. *et al.* (2005) Extracellular like-gregarine stages of *Cryptosporidium parvum*. *Acta Trop.* 95, 74–78

28 Levine, N.D. (1985) Phylum II. Apicomplexa Levine 1970. In *Illustrated Guide to the Protozoa* (Lee, J.J. *et al.*, eds), pp. 322–374, Society of Protozoologists

29 Leander, B.S. *et al.* (2006) Phylogeny of marine gregarines (Apicomplexa) – *Pterospora, Lithocystis* and *Lankesteria* – and the origin(s) of coelomic parasitism. *Protist* 157, 45–60

30 Soldati, D. *et al.* (2004) Molecular and functional aspects of parasite invasion. *Trends Parasitol.* 20, 567–574

31 Dionisio, D. (2002) Cryptosporidiosis in HIV-infected patients. *J. Postgrad. Med.* 48, 215–216

32 Manabe, Y.C. *et al.* (1998) Cryptosporidiosis in patients with AIDS: correlates of disease and survival. *Clin. Infect. Dis.* 27, 536–542