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Author for correspondence:
Virginia Marugan-Hernandez,
E-mail: vhernandez@rvc.ac.uk

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Life cycle stages, specific organelles and invasion mechanisms of *Eimeria* species

Alana Burrell[1,2], Fiona M. Tomley[1], Sue Vaughan[2] and Virginia Marugan-Hernandez[1]

[1]The Royal Veterinary College, University of London, Hawkshead Lane, North Mymms AL9 7TA, UK and [2]Department of Biological and Medical Sciences, Oxford Brookes University, Gipsy Lane, Oxford OX3 0BP, UK

Abstract

Apicomplexans, including species of *Eimeria*, pose a real threat to the health and wellbeing of animals and humans. *Eimeria* parasites do not infect humans but cause an important economic impact on livestock, in particular on the poultry industry. Despite its high prevalence and financial costs, little is known about the cell biology of these 'cosmopolitan' parasites found all over the world. In this review, we discuss different aspects of the life cycle and stages of *Eimeria* species, focusing on cellular structures and organelles typical of the coccidian family as well as genus-specific features, complementing some 'unknowns' with what is described in the closely related coccidian *Toxoplasma gondii*.

Introduction

Eimeria species are protozoan parasites belonging to Coccidiasina (Coccidia) (Fig. 1), a group of obligate intracellular parasites of great human and veterinary importance (Shirley *et al.*, 2005). All members of the Coccidia replicate within the intestines of a definitive host progressing through sequential rounds of asexual (schizogony) and sexual (gametogony) reproduction, culminating in the production of oocysts that are shed into the environment with the feces (Kemp *et al.*, 2013). Coccidians of the family *Eimeriidae*, such as species of *Eimeria* and *Cystoisospora*, are monoxenes meaning that their development is restricted to a single host where they replicate rapidly to reach high numbers in the intestine causing acute enteritis of varying severity. This is in contrast to coccidians of the family *Sarcocystidae*, such as *Toxoplasma gondii* and *Neospora caninum*, which are heteroxenes that complete their whole cycle within the intestines of a definitive host and can also undergo asexual replication within a variety of intermediate host species. Here, infection progresses through an acute, rapid-replication phase into a chronic phase where parasites persist as cysts, most commonly in brain and muscle tissues (Wohlfert *et al.*, 2017). Heteroxene Coccidia may also form tissue cysts in the definitive host where they remain dormant throughout the life of the host, but can reactivate into the acute phase and can cause a range of diseases including encephalitis and abortion.

In contrast to the very broad host range of *T. gondii* (Cowper *et al.*, 2012) species of *Eimeria* are highly host adapted and are generally capable of parasitizing a specific intestinal location in a single host (Yun *et al.*, 2000*a*; Augustine, 2001; Cowper *et al.*, 2012). In Dobell's paper titled 'The Discovery of the Coccidia' (Dobell, 1922) he concludes that it was Leeuwenhoek who first described an *Eimeria* specie some 200 years before the conception of the genus by Schneider in 1875 (Schneider, 1875): in one of his letters in 1674, Leeuwenhoek describes numerous microscopic globules in the bile of rabbits which Dobell believes must have been oocysts of *Eimeria stiedae*. In 1879, Leuckart founded the class Sporozoa, grouping together the Coccidia and similar organisms which are encased within a protective 'spore' covering (Leuckart, 1879). In the early 20th century, light microscopists were able to describe in detail the life cycles of *Eimeria* spp. and reveal their intimate connection with the tissues of the host (Fantham, 1910). By the late 90s many electron microscopy studies had been performed, characterizing organelle and cytoskeletal make-up of *Eimeria* spp. (Ryley, 1969). Electron microscopy was also instrumental to understanding how apicomplexans enter and reside within host cells (Scholtyseck, 1965; Sheffield and Hammond, 1966; Aikawa *et al.*, 1978).

Eimeria is a large genus, with over 1800 species identified to date (Duszynski, 2001). Despite exquisite host specificity of individual species, the genus as a whole has a highly diverse host range and affects members of all vertebrate classes (Duszynski, 2001). Interestingly, one host not affected by *Eimeria* is *Homo sapiens* (Relman *et al.*, 1996). Humans are however specifically infected by *Cyclospora cayetanensis*, a parasite which is highly similar to *Eimeria* species in terms of both genetics and pathogenesis (Liu *et al.*, 2016) and is currently considered as the 'human *Eimeria*'. As the replication stages of schizogony and gametogony occur within host cells, infection with *Eimeria* species results in cellular destruction and pathology to the susceptible host. The usual site of this replication is within epithelial cells lining the intestinal tract. This can lead to clinical symptoms of gastrointestinal dysfunction such as diarrhoea, dehydration and failure to gain weight (Yun *et al.*, 2000*b*). The diseases caused by *Eimeria* spp. are commonly known as 'coccidiosis' or in some cases 'eimeriosis'.

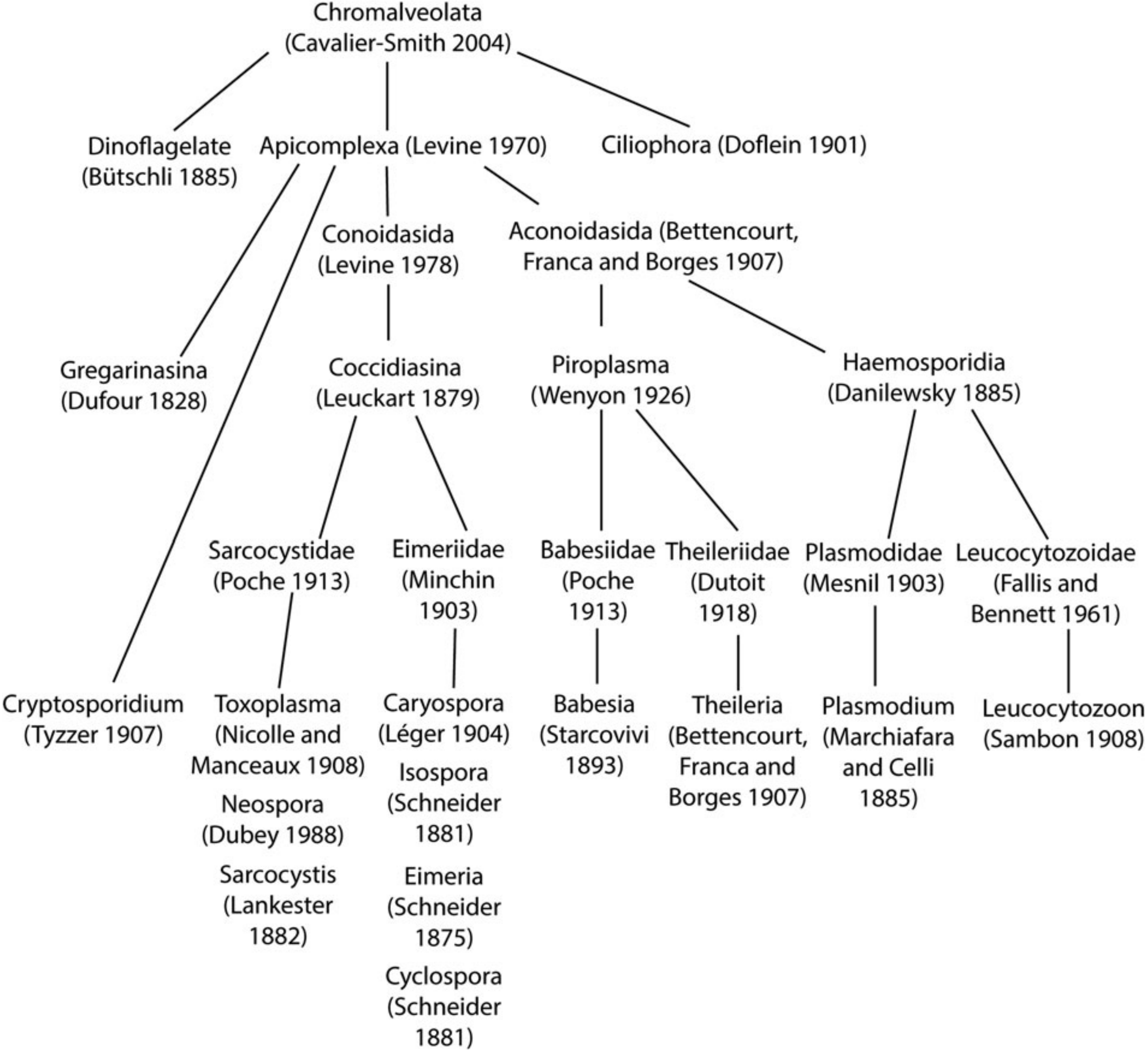

Fig. 1. Phylogenetic tree showing the relationships between relevant apicomplexan species. The tree was generated based on data from the following sources: Levine, 1984; Tenter and Johnson, 1997; Carreno *et al.*, 1999; Jirku *et al.*, 2002; Tenter *et al.*, 2002; Adl *et al.*, 2007; Lane and Archibald, 2008; Golemansky, 2015; Megia-Palma *et al.*, 2015.

The following sections predominantly refer to research involving species of *Eimeria*; where data involving *T. gondii* is used, for example where equivalent studies in *Eimeria* species are missing, this is stated within the text.

Chicken coccidiosis

The most economically important disease caused by *Eimeria* species is coccidiosis in chickens. The estimated global cost of this disease, as stated in a review by Peek and Landman, is more than 2 billion US dollars per year through production losses and costs associated with treatment and prevention measures (Peek and Landman, 2011). There are seven recognized species of *Eimeria* affecting chickens: *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella* (Shirley *et al.*, 2005, 2007). Previously described species *E. mivati* and *E. hagani* (Edgar and Seibold, 1964) (Levine, 1938) are regarded as *nomina dubia* by the vast majority of coccidiologists and to our knowledge there are no isolates of these available upon which definitive molecular tests could be applied. There are however a number of hitherto unclassified isolates, referred to as operational taxonomic units X, Y and Z that are widely distributed throughout the world and may represent novel cryptic species (Clark *et al.*, 2016; Jatau *et al.*, 2016). Advances in this area for a final classification would be necessary for accurate control strategies for these isolates.

Each of the seven recognized species has a set of distinct characteristics in terms of prevalence, pathogenicity, site of infection in the intestine and oocyst morphology (Table 1). *Eimeria tenella* specifically targets the paired caeca, often resulting in fairly extensive haemorrhage; the lesions are primarily caused by second generation schizonts that develop deep to the intestinal epithelium within the lamina propria (Fernando *et al.*, 1983). Infection with *E. maxima* is likely to cause a thickening of the intestinal lining, with mucoid to bloody exudate, and *E. acervulina* is described as causing 'ladder-like' white bands across the mucosa. *Eimeria brunetti* and *E. necatrix* are also capable of causing severe pathology, however they are less commonly encountered compared to *E. acervulina*, *E. maxima* and *E. tenella* (Trees, 2001).

The life cycle of *Eimeria* species

All *Eimeria* species infecting chickens exhibit a similar life cycle (Lillehoj and Trout, 1993) (Fig. 2). Unsporulated oocysts are released in the feces of an infected animal and persist in the environment for long periods. When exposed to air, moisture and

Table 1. Comparison of the seven known *Eimeria* species affecting chickens in terms of site of infection, level of pathology and oocyst morphology

Species	Site of pathology	Lesion score	Oocyst morphology (size range = 15–30 μm)
E. tenella	Caeca	High	Medium round
E. maxima	Mid small intestine	Medium	Large oval
E. acervulina	Upper small intestine	Medium	Small oval
E. necatrix	Mid small intestine	High	Small-medium round
E. brunetti	Distal small intestine + colon	High	Medium oval
E. mitis	Upper small intestine	Low	Small round
E. praecox	Upper small intestine	Low	Medium round

warmth, oocysts go through a developmental process called sporulation (Shirley *et al*., 2005) [Fig. 2(1)]. If ingested by a chicken, the sporulated oocyst will release sporocysts [Fig. 2(2)]. As these pass into the small intestine, enzymatic digestion releases the sporozoites, which migrate to their preferred site of development to initiate cellular invasion (Jeurissen *et al*., 1996) [Fig. 2(3)]. The developmental cycle in the host cell begins with two to three rounds of asexual replication known as schizogony. This process involves multiple nuclear divisions to produce a large multinuclear cell called schizont, from which merozoites are formed (Fig. 2.4 and 2.5). After several generations of merozoite production, parasite development proceeds with a single round of sexual replication known as gametogony (Fig. 2.6), forming the dimorphic stages of macrogamete and microgamete. Finally, macrogamete/microgamete fertilization occurs to form a zygote (Ferguson *et al*., 2003). The zygote will then develop into an oocyst, which, after release in the feces, matures into an infective sporulated oocyst (Jeurissen *et al*., 1996; Shirley *et al*., 2005) [Fig. 2(7)].

The infective oocyst

The oocyst, which has important roles in both parasite development and disease propagation (Lillehoj and Trout, 1993), can

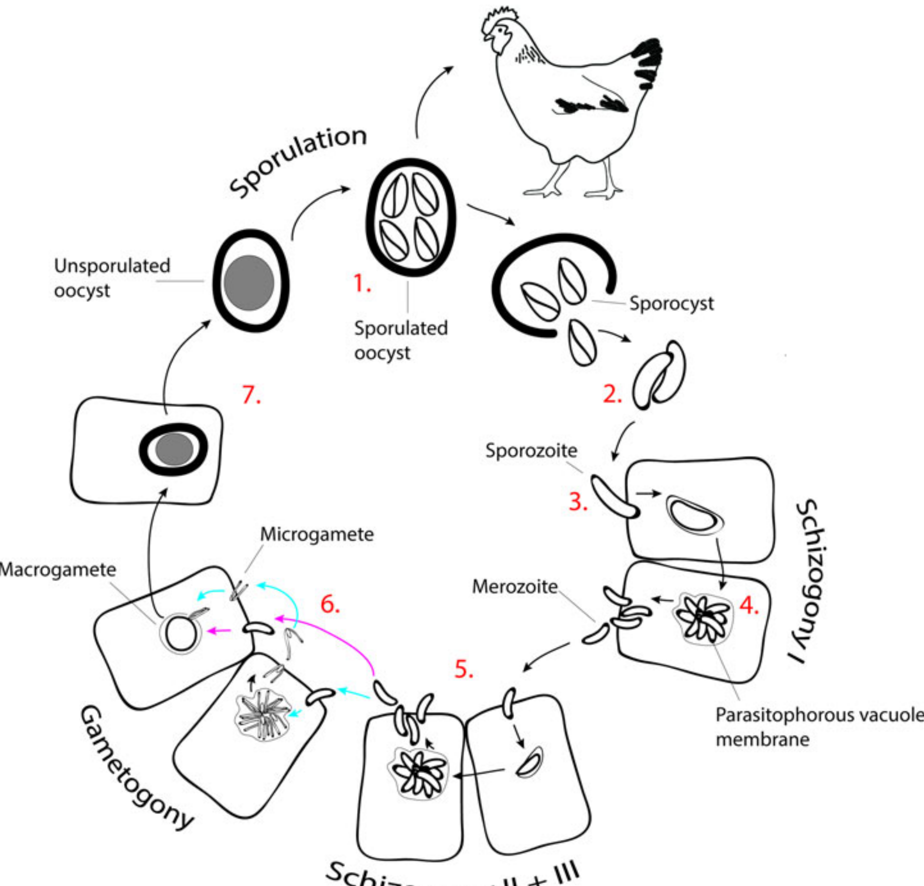

Fig. 2. Life cycle of *Eimeria tenella*. Numbers correlate with subsequent stages of the development. (1) Oocyst sporulation in the environment and oral ingestion by the chicken. (2) Release of sporocysts and sporozoites along the transit in the chicken digestive system. (3) Active invasion of sporozoites in the ceaeca epithelium and formation of the intracellular trophozoite within the parasitophorous vacuole. (4) First round of shyzogony and release of first generation merozoites. (5) Second and third rounds of shyzogony and release of second and third generation merozoites, respectively. (6) Development of microgametes and macrogametes (gametogony) and fecundation. (7) Zygote, development of the oocyst and release to the environment as unsporulated oocyst.

persist in the environment for long periods, even in the presence of disinfectants (Peek and Landman, 2011). This is largely due to protection afforded by the oocyst wall, measuring around 100 nm in thickness and formed from two opposed but easily separated layers and composed of a mixture of lipids and glycoproteins (Stotish *et al.*, 1978; Belli *et al.*, 2006; Mai *et al.*, 2009). The final stage of oocyst development (sporulation) occurs outside of the host and involves a single meiotic division followed by a round of mitosis, resulting in eight infective parasite stages (sporozoites) arranged as pairs inside four individual casings (sporocysts) within each oocyst (Canning and Anwar, 1968) [Figs 2(1) and 3]. As well as containing two sporozoites, each sporocyst contains a micropyle at the apex and a lipid-rich residual body which remains within the sporocyst following sporozoite excystation. The stieda and sub-stieda bodies are found at the anterior pole of the sporocyst, acting as a barrier to sporozoite release until their degradation in the presence of trypsin (Roberts *et al.*, 1970*b*).

The invasive sporozoite

Most knowledge on the sporozoite comes from *E. tenella*, the most extensively studied of the avian *Eimeria* species due to its amenability for sporozoite invasion and schizont development *in vitro*, as well as its potential for genetic manipulation. The *E. tenella* sporozoite is a distinctly polarized cell, of sickle shape and around 10 μm in length (Figs 4A and 5B). Sporozoites contain many classical features and organelles of typical eukaryotic cells such as the nucleus, mitochondrion, endoplasmic reticulum and Golgi apparatus (Fig. 4D and E). They also contain several features specific to the phylum (such as the conoid, apicoplast, micronemes and rhoptries; Figs 4B, C, G and 5A), and even structures which appear to be unique to this genus [refractile bodies (RBs); Figs 4A and 5B]. The *E. tenella* sporozoite nucleus is situated in roughly the centre of the parasite (Fig. 4A), contains a nucleoplasm of fine granular consistency and is surrounded by a pore-containing double membrane (Strout and Scholtyseck, 1970; Pacheco *et al.*, 1975). Adjacent to the nucleus is the Golgi apparatus (Fig. 4D), which contains material described as 'small vesicles in a finely granular matrix' (Vetterling *et al.*, 1973). The mitochondrion of a *T. gondii* tachyzoite, reconstructed from serial electron microscopy data, was found to be a single elongated structure distributed throughout the cell (Melo *et al.*, 2000) (Fig. 5A).

Several early investigations into the structure and composition of the *Eimeria* cell surface revealed the presence of an inner membrane complex (IMC) composed of a meshwork of flattened sacs found directly beneath the outer plasma membrane (Fig. 4F) (Dubremetz, 1975; Dubremetz and Torpier, 1978). Building on the work by Dubremetz (1975) and Dubremetz and Torpier, (1978), studies of *T. gondii* tachyzoites have shown that the IMC is connected to the subpellicular microtubules *via* a network of intermediate filaments and alveolin (Morrissette and Sibley, 2002). They have an important role in maintaining cell rigidity, critical for assembly and stabilization of the actin-myosin motor that powers parasite gliding motility (Sibley, 2010), a point of attachment for organelles (Kudryashev *et al.*, 2010) and apparently originates from the Golgi apparatus (Francia and Striepen, 2014).

Beneath the pellicle there is a set of longitudinally running microtubules. One of the first depictions of this cytoskeletal organization was achieved in *E. acervulina* through 'critical point drying' (a method of dehydrating a biological sample without distorting morphology), allowing the isolation and electron microscopy examination of the outer cytoskeletal microtubules (Russell and Sinden, 1982). More recently, several reviews have

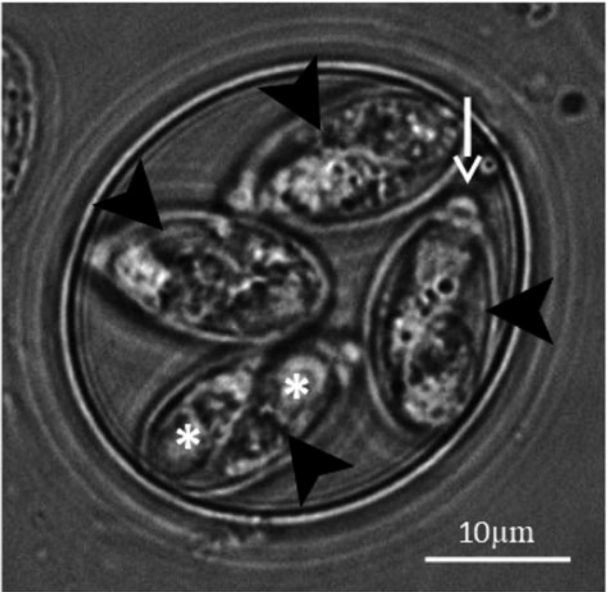

Fig. 3. Sporulated oocysts of *E. tenella*. The oocysts contain four sporocysts (arrowheads), each containing two sporozoites (asterisks) and a micropyle at each sporocysts apex (arrow).

focused on the architecture and role in invasion of the cytoskeleton in coccidian parasites (Morrissette and Sibley, 2002; Frenal and Soldati-Favre, 2009; Sibley, 2010). The 24 subpellicular microtubules of *Eimeria* species are arranged as an evenly spaced spiral and extend to roughly half of the parasite length, acting as a support scaffold to maintain cell shape and rigidity (Vetterling *et al.*, 1973; Russell and Sinden, 1982; Morrissette and Sibley, 2002). The apical ends of these microtubules are fixed in a ring around the conoid, forming the apical polar ring (APR), however it is not clear whether their posterior ends are fixed or free (Vetterling *et al.*, 1973; Russell and Sinden, 1982).

Refractile bodies, amylopectin granules and acidocalcisomes

In light and electron micrographs of *E. tenella* sporozoites, the most striking structures are the RBs (Figs 4A and 5B). In transmission electron micrographs (TEMs) these appear as spherical or ovoid structures with homogeneous electron dense content and no obvious limiting membrane. Usually a spherical RB is found anterior to the nucleus (around 1–2 μm in diameter) and an ovoid RB is found posterior to the nucleus (around 5 μm in length). Despite their striking appearance, the functions of RBs remain unknown; the most commonly hypothesized roles are protein storage and metabolism (de Venevelles *et al.*, 2006). Work by Fayer described an interesting dynamic, whereby after host cell invasion the anterior and posterior RB merge into a single RB that localizes centrally in the schizont (Fayer, 1969). Twenty years later a monoclonal antibody (1209-C2) specific for RBs (and able to bind related *E. tenella* proteins) was used to show that the merged RB separates into globules that diffuse through the schizont and re-concentrate as a refractile 'dot' within each first-generation merozoite (Danforth and Augustine, 1989). The protein target of the 1209-C2 was identified independently by two research groups seeking to validate vaccine antigens for control of coccidiosis (Miller *et al.*, 1989; Crane *et al.*, 1991). This RB protein, termed variously GX3262, 'B' antigen and SO7, contain large numbers of repetitive amino acid tracts (Liberator *et al.*, 1989; Reid *et al.*, 2014) and induce partial protection against challenge with up to four different species of *Eimeria* (Bhogal *et al.*, 1989; Karkhanis *et al.*, 1991). SO7 (most common name) has been used as a candidate antigen in a variety of vaccinology studies (Song *et al.*, 2015; Yang *et al.*, 2017) although the mechanism of immune protection, and the biological function of this family of RB proteins, that are unique to the genus, remain unknown. A few additional proteins have been localized to the RBs including an aspartyl protease (Laurent *et al.*, 1993), a putative nucleotide transhydrogenase (Vermeulen *et al.*, 1993) and some other up-represented in a RB enriched proteome such as a lactate dehydrogenase, a carbonyl reductase, a subtilisin 2 protease and a haloacid dehalogenase-like hydrolase (de Venevelles *et al.*, 2006).

Surrounding the larger (posterior) RB and dispersed throughout the posterior two-thirds of the parasite, are several tens of amylopectin granules (Vetterling *et al.*, 1973; Pacheco *et al.*, 1975) (Fig. 5B). These are smaller than the RBs and easily identified in TEMs, appearing electron dense or lucent dependent on the staining technique (Ryley *et al.*, 1969; Strout and Scholtyseck, 1970; Fernando and Remmler, 1974). Amylopectin is an important carbohydrate source for *Eimeria*, being present in the oocyst, sporozoite and merozoite stages, as well as in the residual body of the schizont (Ryley *et al.*, 1969; Coombs *et al.*, 1997). In *T. gondii*, amylopectin granules are much more numerous in bradyzoites (the latent tissue cyst stage) than tachyzoites (the rapid proliferation stage) suggesting they could be used to fuel transition between these two stages (Guerardel *et al.*, 2005). *Eimeria* species contain another group of organelles that appear by TEM as membrane bound spherical structures roughly half a micron in diameter and filled to varying extents with highly electron dense material. In *T. gondii* analogous structures are classified as acidocalcisomes: acidic organelles containing high concentrations of various physiologically important ions (Miranda *et al.*, 2008; Soares Medeiros *et al.*, 2011) (Fig. 5A).

Conoid

The conoid is a movable cone-shaped structure which sits within the APR, from which the subpellicular microtubules emanate (Ryley, 1969; Vetterling *et al.*, 1973) (Fig. 5). In *T. gondii*, the conoid was found to be composed of a novel tubulin polymer, arranged as in a tight spiral of 'microtubule-like' fibres (Hu *et al.*, 2002). Studies on the molecular composition of the *T. gondii* conoid revealed the presence of several additional novel proteins (Hu *et al.*, 2006), including three calmodulin-like proteins involved in motility, invasion and egress, but not required for conoid extrusion or microneme/rhoptry secretion (Long *et al.*, 2017*b*), as well as an essential protein (CPH1) involved in conoid stability (Long *et al.*, 2017*a*). In several species of *Eimeria*, the conoid has been observed in both extended and retracted states (Roberts and Hammond, 1970). However, in electron micrographs depicting extracellular, invading or intracellular *Eimeria* sporozoites, the conoid is rarely seen in the extended position (Roberts *et al.*, 1970*a*, 1971; Vetterling *et al.*, 1973; Jensen, 1975). In *T. gondii* the conoid has been shown to rotate, tilt, extend and retract (Bommer *et al.*, 1969), often seen in the extended state immediately prior to invasion (Chiappino *et al.*, 1984; Carruthers and Boothroyd, 2007). This range of movement has led to the hypothesis that the conoid has a mechanical role in invasion (Morrissette and Sibley, 2002).

Micronemes and rhoptries

Micronemes are small (20 nm by 50 nm) rod-shaped organelles located at the anterior end of the zoite (Fig. 4B). Their protein content is well defined in several coccidians, including *E. tenella* and *T. gondii.* There are genus-specific differences in the precise portfolio of MICs but overall these are generally conserved, comprising a mix of soluble and membrane-spanning proteins, many with domains orthologous to known adhesins (reviewed by Tomley and Soldati, 2001; Carruthers and Tomley, 2008;

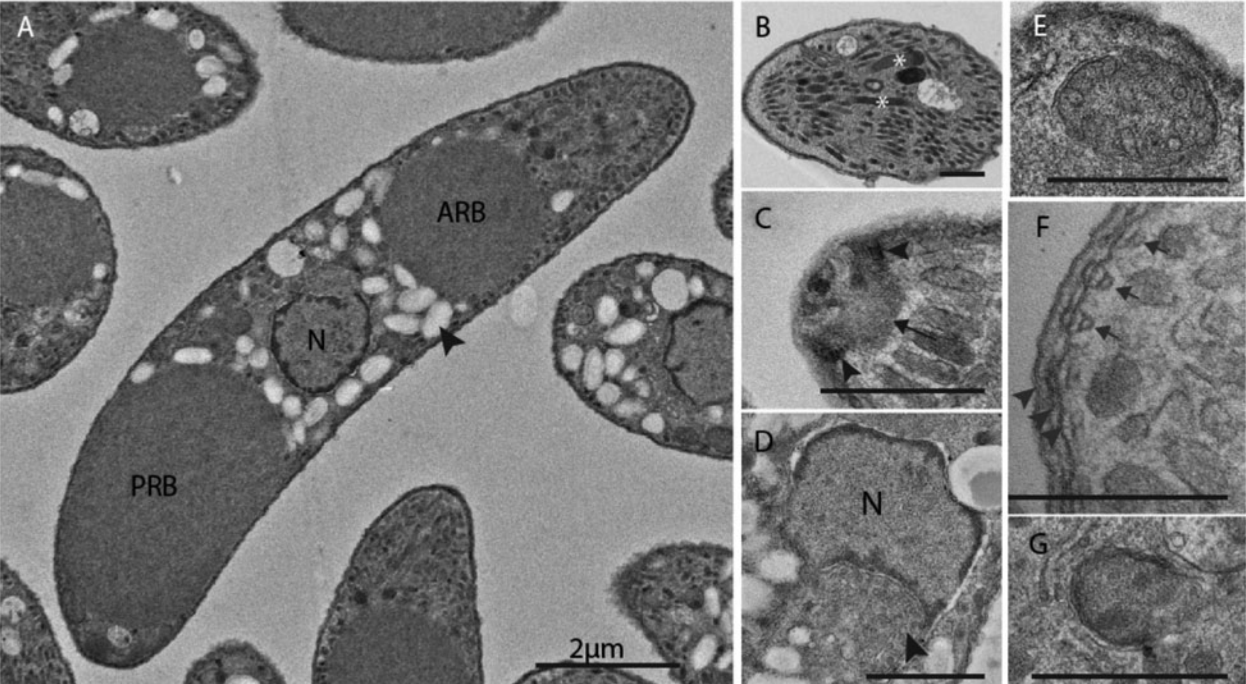

Fig. 4. TEMs of *E. tenella* sporozoite ultrastructures (A. Burrell, unpublished). (A) Sporozoites with two large non-membrane bound organelles known as anterior and posterior refractile bodies (ARB and PRB) situated at either side of the nucleus (N) as well as numerous amylopectin granules (arrowhead). (B) Micronemes and rhoptries (asterisks) occupying most of the cytoplasm in the anterior quarter of the cell. (C) Apex of the cell with a cone-shaped structures composed of helical fibres known as the conoid (arrow) sitting within the APRs (arrowheads). (D) Centrally located nucleus (N) next to which the Golgi apparatus can be observed (arrowhead). (E) Mitochondrion cross-section showing plump cristae. (F) Triple-layered pellicle (arrowheads) consisting of plasma membrane and IMC beneath which sits an array of sub-pellicular microtubules (arrows). (G) Apicoplast with four membrane layers. Scale bars: (A) ~2 μm; (B–G) ~500 nm.

Cowper *et al.*, 2012). Surface-expressed MICs are able to bind host ligands (essential for gliding motility, attachment and invasion of host cells) and also bind parasite-derived RON ligands to form the moving junction (discussed later).

Rhoptries are club-shaped organelles (up to around 2000 nm in length) often seen with the narrow neck portion extending longitudinally into the conoidal channel (Fig. 5A and B). It is not clear precisely how many rhoptries each sporozoite contains, although up to eight have been seen for *E. tenella* (Pacheco *et al.*, 1975). Rhoptry proteins are housed in two compartments: RONs are stored in the rhoptry necks and ROPs come from the bulbous part of the organelle (Besteiro *et al.*, 2011).

Biogenesis of the microneme and rhoptry

The apical organelles are part of the parasite endomembrane system and in *E. tenella* are generated *de novo* late in the formation of sporozoites (Ryan *et al.*, 2000) and merozoites (Brown *et al.*, 2000; Tomley and Soldati, 2001). From studies in *T. gondii* and *Plasmodium* species it is well established that apicomplexans have re-purposed the exocytic and endocytic pathways of higher eukaryotes into a partially 'merged' vesicle and protein trafficking system in order to generate their specialized regulatory secretory organelles (reviewed by Tomavo *et al.*, 2013; McGovern and Carruthers, 2016). Microneme (MIC) and rhoptry (ROP/RON) proteins enter the early exocytic pathway at the endoplasmic reticulum and traffic to the Golgi and trans-Golgi network (TGN), from where they are sorted to novel endosome-like compartments (ELC) bearing early (Rab5) or late (Rab7) endosome markers. In *T. gondii*, over-expression of Rab5A/5C causes defective trafficking of some (but not all) MIC and all ROP/RON proteins presumably by saturating receptors and dysregulating vesicle transport (Kremer *et al.*, 2013).

Several additional molecules are essential for TGN to ELC trafficking of MIC and ROP/RON proteins in *T. gondii* and as gene orthologues are present in *Eimeria* species (www.toxodb.org) it is likely that this trafficking pathway is conserved across the Coccidia. These molecules include vacuolar sorting protein 9, an activator of Rab5 (Sakura *et al.*, 2016); syntaxin 6 (Stx6), a SNARE found mainly in the TGN (Jackson *et al.*, 2013); dynamin-related protein B (DrpB) and clathrin, both found in the TGN and ELC (Breinich *et al.*, 2009; Pieperhoff *et al.*, 2013); the clathrin adaptor complex AP1 (Venugopal *et al.*, 2017); the sortilin-like receptor TgSORTLR (Sloves *et al.*, 2012) and several components of CORVET (class C core vacuole/endosome tethering) and HOPS (homotypic fusion and vacuolar protein sorting) complexes associated with early and late endosomes respectively (Morlon-Guyot *et al.*, 2015, 2018). TgSORTLR loads MIC and ROP/RON proteins at the TGN and escorts them to the ELC (Sloves *et al.*, 2012; Sangare *et al.*, 2016). In higher eukaryotes, sortilin is a transmembrane endosomal receptor with a major role in anterograde transport of lysosomal enzymes from TGN to endosomes (Bonifacino and Rojas, 2006), and in retrograde trafficking where it binds retromer, an evolutionarily conserved protein complex that selects and recycles proteins from endosomes to the TGN or the plasma membrane (Pan *et al.*, 2017). Recent characterization of retromer interactomes in *T. gondii* confirms that retromer-dependent retrograde transport is essential for apical organelle biogenesis, probably because recycling of TgSORTLR to the TGN is needed to maintain anterograde trafficking of MIC and ROP/RON proteins to the ELC (Sangare *et al.*, 2016).

Because not all MIC proteins depend on Rab5A/5C trafficking, it is suggested there is more than one trafficking pathway leading to either distinct populations of micronemes containing different MICs, or a single population having different sub-compartments (Kremer *et al.*, 2013). More evidence for dual trafficking emerged recently with the observation that vacuolar protein sorting 8 (TgVps8, a CORVET component), whilst essential for organelle biogenesis, completely blocks trafficking of most MICs but only

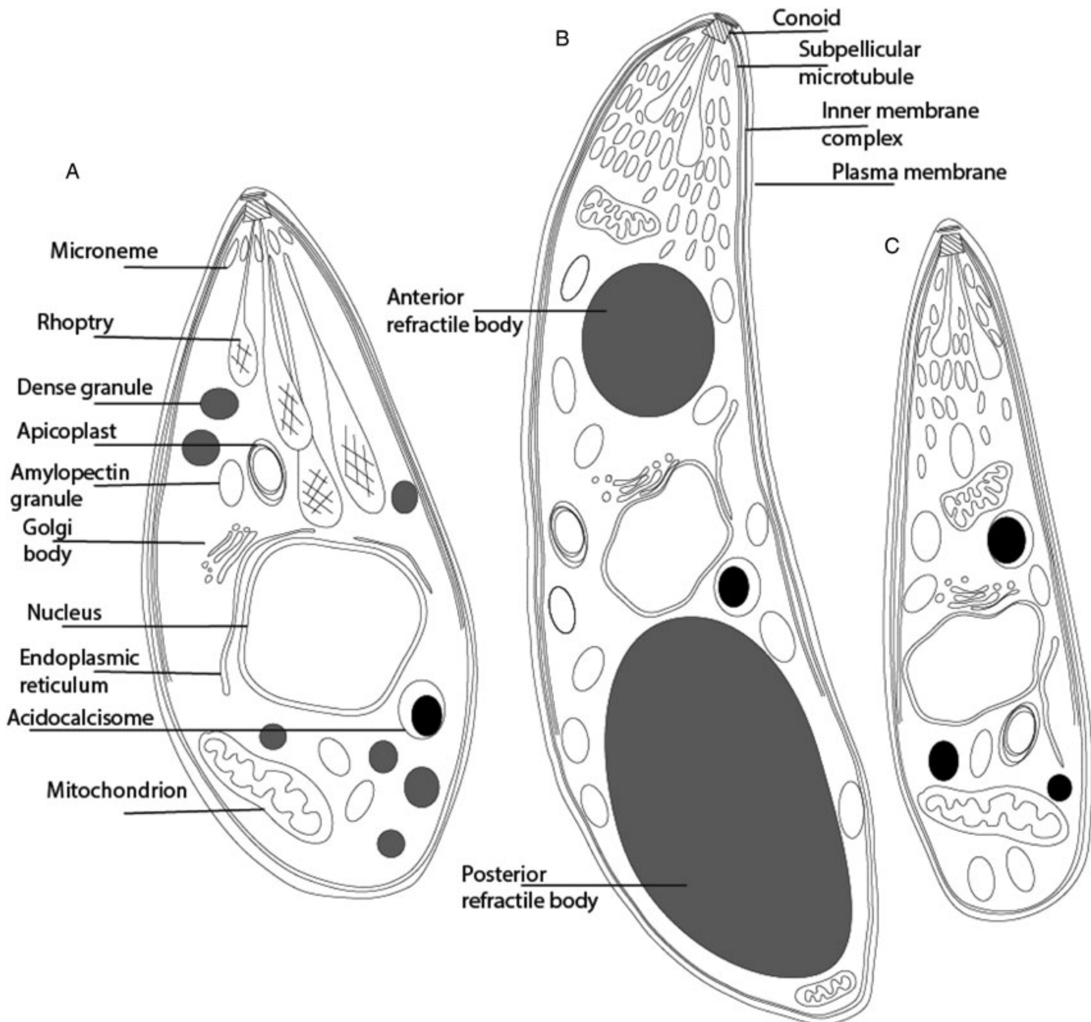

Fig. 5. Cell structure and organelle content of different coccidian cells. (A) Tachyzoite of *Toxoplasma gondii*. (B) Sporozoite of *Eimeria* species. (C). Merozoite of *Eimeria* species.

partially affects that of TgAMA1, TgMIC2 and TgMIC6 (Morlon-Guyot *et al*., 2018). No work on MIC trafficking has been carried out in *Eimeria* species, but lack of co-localization of MIC proteins has been observed, for example between EtMIC3/EtAMA1, and between EtMIC3/EtMIC5 in sporozoites (Lai *et al*., 2011), and between EtMIC2/EtAMA2 in second generation merozoites (McGovern *et al*., 2018; Pastor-Fernandez *et al*., 2018). These studies used non-quantitative imaging and whilst strongly suggestive that zoites contain distinct populations of micronemes, it cannot be ruled out that they reflect differences in timing and levels of individual MIC protein expression.

By merging and adapting exocytosis and endocytosis, apicomplexans have conserved an endomembrane system that is much reduced compared to higher eukaryotes, but efficient to perform the protein trafficking, targeting, processing and re-cycling needed for their obligate intracellular lifestyles. How MIC and ROP/RON proteins are differentially sorted and trafficked beyond the ELC to their target organelles, and how the endomembrane system efficiently segregates exocytosis from endocytosis are major questions. In *T. gondii* divergence between MIC and RON/ROP proteins at the ELC has been noted, with immature MICs (TgM2AP and TgMIC5) located in ELC that also contain endocytosed host protein whilst an immature RON (TgRON4) is in ELC lacking ingested host protein (McGovern *et al*., 2018). Thus endocytosis appears to intersect directly with MIC exocytosis and there must be a mechanism for directing MICs from ELC to micronemes and away from ingested host proteins that are *en route* to the parasite lysosome-like vacuole (VAC, Dou *et al*., 2014). In contrast, trafficking of RON/ROP proteins appears to avoid contact with endocytosed host protein, proceeding *via* an immature pro-rhoptry compartment (McGovern *et al*., 2018). Nevertheless, successful rhoptry biogenesis requires the endosomal CORVET protein TgVps9, and a novel parasite BEACH domain-containing protein that is also essential for VAC formation (Morlon-Guyot *et al*., 2018).

An essential step in late exocytosis of MIC and RON/ROP proteins is proteolytic processing (Nishi *et al*., 2008) that most likely occurs within, or during exit from the ELC (McGovern *et al*., 2018) mediated by proteinases that include aspartyl protease 3 (Dogga *et al*., 2017). Failure to undergo proteolytic maturation results in impaired organelle formation. Interestingly, apicomplexans possess a family of four phylogenetically related transporters belonging to the major facilitator superfamily and termed transporter family protein 1–4 (TFP1–4) (Besteiro *et al*., 2011; Hammoudi *et al*., 2018). In *T. gondii* TFP1 localizes to micronemes and ELC and is critical for condensation of microneme content, presumably by allowing the transport of molecules that are essential for this process such as maturases, or chaperones. Knock down of TFP1 impairs microneme formation and completely blocks MIC exocytosis; TFP2 and 3 localize to the rhoptries and knock down of TFP2 results in elongated rhoptries, again suggesting that defects in condensation/compaction have an impact on the late stages of organelle biogenesis (Hammoudi *et al*., 2018).

Zoite invasion of host cells

A widely researched aspect of coccidian biology is the mechanism by which zoites (sporozoites, merozoites, tachyzoites and bradyzoites) invade the host cell to occupy a unique intracellular niche, the PV. Much of the molecular detail of this process has been described in the *T. gondii* tachyzoite, a model cell for coccidian parasites. Considering the biological differences between tachyzoites and sporozoites (tachyzoites are formed when a sporozoite or bradyzoite stage converts within the cells of a host, rapidly replicates and disseminates throughout the host, then converts back into bradyzoites) (Dubey *et al.*, 1998), we must be careful when inferring knowledge from *T. gondii* tachyzoites to *Eimeria* where there is no such stage-conversion (to tachyzoite or bradyzoite) or persistent infection. However, at the genomic and cellular levels much of the complex invasion machinery used by the coccidia is conserved so it is useful to supplement rather sparse data on *Eimeria* species with knowledge from *T. gondii*.

The process of invasion

Following initial contact with a host cell *in vitro*, the *E. tenella* sporozoite glides across the cell surface in a helical motion, possibly in a search of an appropriate location to invade (Russell and Sinden, 1981; Entzeroth *et al.*, 1989). Before invasion, the sporozoite re-orientates itself so that the apical tip, at which the conoid is located, makes contact with the host cell plasma membrane forming a 'moving junction' (MJ) between parasite and host cell membranes through which the parasite propels itself to enter the newly forming intracellular vacuole. As the parasite pushes itself into the cell, it causes an invagination of the membrane. The MJ remains fixed at the point of attachment to the host cell but is translocated backwards over the surface of the invading parasite from apex to posterior. This invagination continues until the whole parasite length has passed through the MJ, at which point the host membrane pinches together behind the parasite posterior enclosing the parasite within a PV. This process has been well documented in several species of *Eimeria* as well as *T. gondii* and other apicomplexans (Suss-Toby *et al.*, 1996; Entzeroth *et al.*, 1998; Beyer *et al.*, 2002). According to this model, the parasite does not enter the host cell cytoplasm, although there is evidence that some of the rhoptry content enters in the form of e-vacuoles (Hakansson *et al.*, 2001), a process that may be critical for the early release of rhoptry neck proteins (RONs) and formation of the MJ (Besteiro *et al.*, 2011). In addition to stabilizing the site of invasion, the MJ has a role as a molecular sieve, removing non-Glycosylphosphatidylinositol (GPI)-anchored host membrane proteins from the newly formed PV membrane, including the key immune signalling/effector molecules MHC class I, MHC class II and FcR (Mordue and Sibley, 1997). The PV of *T. gondii* differs from phagosomes in that it does not acquire the host-derived proteins involved in endosome fusion thereby protecting the parasite from lysosomal destruction (Mordue and Sibley, 1997; Mordue *et al.*, 1999; Beyer *et al.*, 2002). Although assumed to be similar, the fusion capacities of the PV harbouring *Eimeria* species has not yet been demonstrated (Entzeroth *et al.*, 1998).

Signalling pathways involved in invasion

Waves of regulated protein secretion from the microneme (MIC) and rhoptry (ROP/RON) apical organelles are essential for parasite movement, invasion, formation of the intracellular parasitophorous vacuole, control of host gene expression and egress of daughter zoites from infected cells. These processes are key virulence determinants for most species of the Apicomplexa (Dubremetz *et al.*, 1998; Keeley and Soldati, 2004; Besteiro *et al.*, 2011).

Rapid secretion and surface capping of microneme proteins (MICs) from the apical tip of *E. tenella* sporozoites is induced when sporozoites are allowed to glide over a substrate and during invasion of host cells in cell culture (Bumstead and Tomley, 2000). In the absence of host cells, secretion and capping can be induced by exposure of freshly purified sporozoites to serum, or purified albumin at temperatures of 37 or 41 °C (Brown *et al.*, 2000; Bumstead and Tomley, 2000). At lower temperatures or in the absence of serum or albumin, no MIC secretion or capping is detected. Both parasite invasion and albumin-induced MIC secretion is blocked in *E. tenella* by treatment with a compound that directly inhibits protein kinase G (Wiersma *et al.*, 2004) indicating the likely importance of cyclic GMP (cGMP) signalling in coccidian secretion. PKG-dependent microneme secretion has also been shown in *T. gondii* (Brown *et al.*, 2000) and using a novel auxin-inducible degron tagging system for conditional protein depletion in *T. gondii* alongside CRISPR-Cas9 genome editing, signalling was shown to go through PKG^I, a myristoylated isoform of PKG localized at the parasite plasma membrane (Brown *et al.*, 2017). In addition to cGMP signalling, it is known that calcium (Ca^{2+}) fluxes provide crucial signals for gliding motility, microneme secretion, conoid extrusion, invasion and egress (Lourido *et al.*, 2010; Pu and Zhang, 2012). These pathways operate through specific members of a calcium-dependent protein kinase (CDPK) family that is conserved in *Eimeria* (Dunn *et al.*, 1996). A detailed chemical genetics (mutation) approach shows that the pathways linked to parasite invasion and egress, and the secretion of specific MIC proteins are differentially controlled by different CDPKs, and intersect cGMP signalling (Besteiro *et al.*, 2011; Lourido *et al.*, 2012). A variety of treatments that cause transient fluxes of cytosolic Ca^{2+} induce MIC secretion in *T. gondii* tachyzoites including calcium ionophores (Carruthers and Sibley, 1999), ethanol and acetaldehyde, but this is dependent upon the presence of albumin and cGMP signalling (Brown *et al.*, 2016). Similarly in *E. tenella*, acetaldehyde and ethanol stimulate Ca^{2+}-dependent MIC secretion and premature egress of sporozoites from cultured cells in the presence of serum (Yan *et al.*, 2015, 2016), however in the absence of serum or albumin neither ionophores nor ethanol/acetaldehyde are effective (F. M. Tomley and J. M. Bumstead, unpublished). Thus it appears that cGMP and Ca^{2+}-signalling pathways work co-operatively in MIC signalling, with PKG^I at the plasma membrane being the essential 'master' regulator (Brown *et al.*, 2017) whilst members of the CDPK family provide the selectivity and specificity needed to carry out specific biological functions such as invasion or egress.

Signalling pathways leading to the exocytosis of rhoptry contents are not yet defined however these must allow the selective secretion of RON proteins early in readiness for their role in formation of the MJ (Besteiro *et al.*, 2011). It has also been reported that *T. gondii* is able to inject ROP proteins into host cells that it does not invade, allowing the parasite to manipulate uninfected cells (Koshy *et al.*, 2012) suggesting that e-vacuole (Hakansson *et al.*, 2001) deployment is an important virulence factor.

The role of micronemes and the glideosome

Microneme proteins (MICs) are secreted from the parasite apex either singly or as protein complexes onto the parasite surface (Tomley *et al.*, 1996; Brown *et al.*, 2000; Bumstead and Tomley, 2000; Lai *et al.*, 2009) a process mediated in *T. gondii* by DOC2 proteins that recruit the necessary membrane-fusion machinery (Farrell *et al.*, 2012). 'Capping' models of motility, whereby parasite molecules are rapidly translocated backwards over the surface to promote forward motion, were proposed over 40 years ago for

Plasmodium (circumsporozoite precipitation reaction, Vanderberg, 1974), *Eimeria nieschulzi* (capping of ferritin, Dubremetz and Torpier, 1978) and gregarines (capping of conA-coated latex beads, King, 1981). The importance of the actin in motility was recognized in both *Eimeria* (Jensen and Edgar, 1976; Russell and Sinden, 1982) and *Plasmodium* (Miller *et al.*, 1979) and a later study in *E. tenella* showed that material secreted when sporozoites were allowed to glide on a substrate emanated from the apical tip (Entzeroth *et al.*, 1989). Subsequently, a large number of studies, mainly in *T. gondii*, has led to definition of 'glideosomes' (Opitz and Soldati, 2002), protein complexes that lie between the parasite plasma membrane and the IMC and power substrate-dependent gliding motility (reviewed in detail by Frenal and Soldati-Favre, 2009).

In brief, binding of surface-bound MIC adhesins to host ligands provides traction, linkage of these parasite–host surface membrane complexes to the underlying action-myosin motor is needed for their translocation (capping). This is achieved by the glideosome-associated connector (GAC), an armadillo repeat-containing protein that accumulates under the plasma membrane at the apical tip and stabilizes freshly polymerized short F-actin filaments that are nucleated at the tip by parasite formins (Jacot *et al.*, 2016). GAC binds directly to the cytosolic tails of surface-bound transmembrane MICs (Jacot *et al.*, 2016) and in a two-stage process, stabilized actin-GAC-MIC complexes are rapidly shuttled backwards through the interaction of the actin tracks initially with MyoH glideosomes, that are restricted to the conoid region (Graindorge *et al.*, 2016), and thereafter with MyoA glideosomes positioned along the length of the zoite (Herm-Gotz *et al.*, 2002). The complexes are shed from the posterior of zoites by the action of an intramembrane rhomboid-like serine protease, ROM4, which cleaves MIC transmembrane spanning regions (Buguliskis *et al.*, 2010). To generate the force needed for forward motion, glideosomes need to be linked fluidly at the parasite plasma membrane and immobilized onto the cytoskeleton, a feat achieved by glideosome protein GAP45 which has its acylated N-terminus embedded in the plasma membrane and its C-terminus cross-linked to the IMC (Gilk *et al.*, 2009). Additional glideosome proteins GAP40 and GAP50 are further involved in anchoring MyoA firmly the IMC (Harding *et al.*, 2016) and a family of multi-membrane spanning GAPM proteins connect the glideosome right through to the subpellicular microtubules, *via* interaction with alveolins (Bullen *et al.*, 2009). The regular positioning and complex molecular architecture of the glideosomes suggests that these structures are equivalent to the intramembrane particles visible in scanning electron micrographs of freeze-fractured IMC from sporozoites of *Eimeria* taken over 40 years ago (Dubremetz and Torpier, 1978).

By virtue of their host-binding activity, MICs are major contributors to parasite host-range and specificity; for example, MAR (microneme adhesive repeat)-domain containing MICs of *E. tenella* contain a single type (type 1) of MAR (Lai *et al.*, 2011) whereas *T. gondii* and *N. caninum* possess MICs with both type 1 and 2 MAR. MARs bind sialyl-terminated oligosaccharides from many types of vertebrate tissue so expressing only a single type effectively narrows the range of sialylated receptors that *E. tenella* can bind, contributing to the very specific tropism of this parasite (Cowper *et al.*, 2012). In *T. gondii* secretion of perforin from micronemes is essential for tachyzoite egress from vacuoles (Roiko and Carruthers, 2013). A role for perforin in egress has not been confirmed for *Eimeria* parasites; a gene encoding a conserved membrane-attack complex/perforin is expressed in *E. tenella* sporozoites but appears to be down-regulated in the later merozoite and gamete stages (Reid *et al.*, 2014; Walker *et al.*, 2015).

Secretion of rhoptries and dense granules proteins

RONs (rhoptry neck protein) and ROPs (rhoptry bulb proteins) are believed to discharge from the apex of the parasite; rhoptry ducts run through the conoid and terminate at the very apical tip. In *T. gondii*, RONs act in concert with apical membrane antigens (AMA, secreted from the micronemes) at the early stage of invasion, assembling at the parasite–host interface to form the irreversible MJ (Besteiro *et al.*, 2011; Lamarque *et al.*, 2011; Tyler and Boothroyd, 2011) actively recruiting host proteins to the MJ, subverting their function to enhance invasion efficiency (Guerin *et al.*, 2017). Proteomic and genomic analysis readily identified several families of RONs in *E. tenella* orthologous to those of *T. gondii*, along with stage-regulated expression of specific AMA and EtRON2 family members (Oakes *et al.*, 2013), suggesting that the mechanism by which the MJ is built by different coccidians is conserved. In contrast there is only limited conservation of ROPs between *T. gondii* and *E. tenella* including significant divergence in the families of ROP kinases that are the major component of the ROP proteome (Oakes *et al.*, 2013; Talevich and Kannan, 2013) and which are known to be key virulence factors in *T. gondii*. Little is known of the specific function of individual ROP proteins in *Eimeria*. Among the *Eimeria* species affecting chickens, rhoptry proteins offer little immunological cross-reactivity between the various species or even between different life cycle stages within the same species (Kawazoe *et al.*, 1992; Tomley, 1994).

Another group of secretory organelles related to host cell interactions in *T. gondii*, and other heteroxenic, cyst-forming coccidian, are the dense granules. These are roughly spherical structures larger than micronemes but smaller than rhoptries (Paredes-Santos *et al.*, 2012). The contents of dense granules are secreted into the PV during and immediately after invasion, and dense granule proteins (GRA) are targeted to a variety of final locations including the PV cavity, PV membrane, host cell cytoplasm and host cell nucleolus (Mercier and Cesbron-Delauw, 2015). However, the presence of dense granules as an independent organelle in the zoites of *Eimeria* spp. is uncertain as there is a lack of structural evidence (Vetterling *et al.*, 1973; Entzeroth *et al.*, 1998) and moreover only a very small number of GRA orthologues are found in their genomes (Reid *et al.*, 2014).

Post-invasion events

For *Eimeria* species, the newly-formed PV is small and closely surrounds the parasite but later enlarges, possibly contain membranous material or projections from the vacuolar membrane (Strout and Scholtyseck, 1970; Lee and Long, 1972; Vetterling *et al.*, 1973; Pacheco *et al.*, 1975). In electron micrographs of recently invaded sporozoites and merozoites, the PV is often not visible. It is unclear whether this is because there is no vacuole present or because the vacuole membrane is so closely opposed to the parasite that it cannot be distinguished (McLaren, 1969; Lee and Long, 1972; Mota and Rodriguez, 2001). Between 24 and 35 h after invasion, the intracellular sporozoite becomes ovoid in shape [Fig. 6(1)]. At this stage, the parasite is known as a trophozoite and loses most of its apical complex and inner membrane (McLaren, 1969; Pacheco *et al.*, 1975).

Fairly soon following inoculation of *in vitro* cell cultures, some *Eimeria* species sporozoites have been seen to leave their invaded host cell without undergoing further development and replication. It has been hypothesized that some of this cell traversal may involve penetration through the host cell plasma membrane rather than formation of a PV (Itagaki *et al.*, 1974; Behrendt *et al.*, 2004). This hypothesis is supported by the observation that *Plasmodium yoelii* sporozoites will sometimes invade hepatocytes by breaching the host cell membrane (Mota *et al.*, 2001). Breaching of the host cell membrane has been described for *E. bovis* (Behrendt *et al.*,

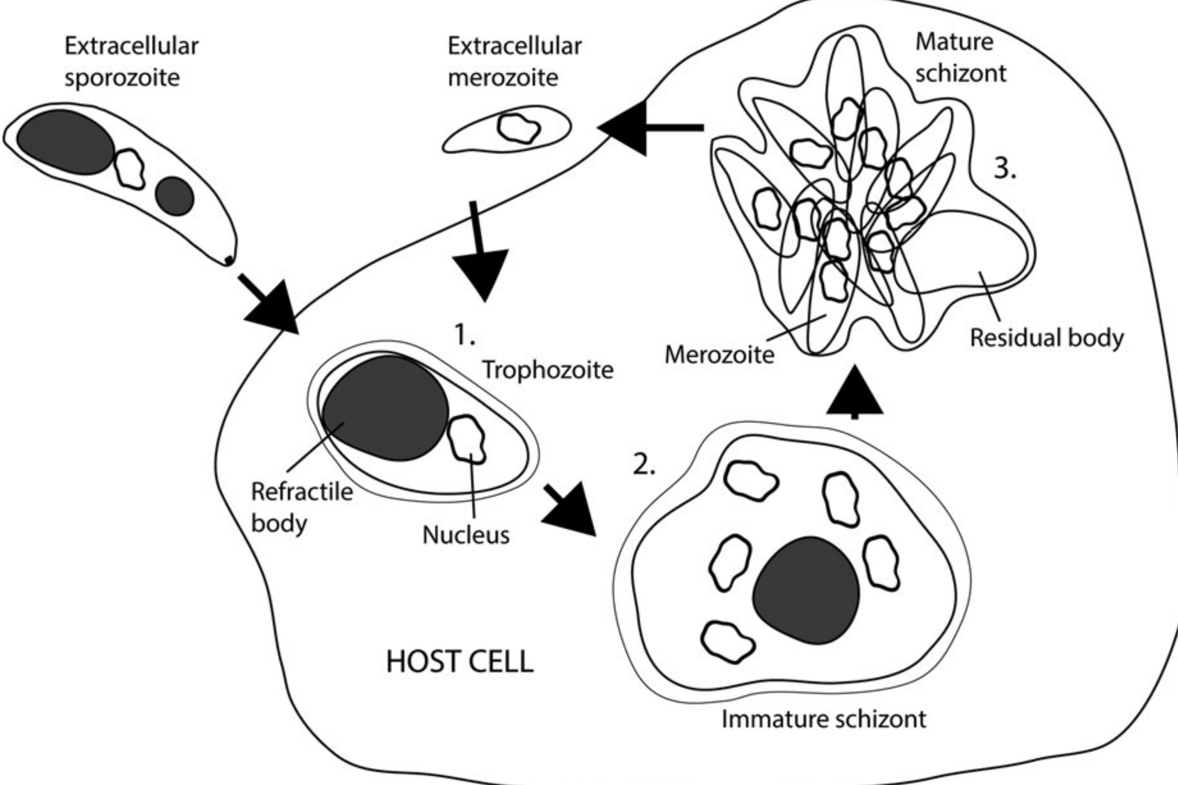

Fig. 6. Schizogony (adapted from Francia and Striepen, 2014). (1) Trophozoite development after sporozoite invasion. (2) Immature schizont, nuclei multiply by several rounds of mitosis. (3) Mature schizont, the last round of division coincides with the merozoites budding at the parasite surface. Merozoites release and initiate a new round of schizogony (or gametogony).

2004), however studies using *E. magna* demonstrated no breach to plasma membrane on parasite invasion (Jensen, 1975; Jensen and Edgar, 1976). This suggests that invasion of cells by *Eimeria* species does indeed follow the generally accepted model for apicomplexan invasion.

Initial invasion of *E. acervulina*, *E. maxima*, *E. necatrix* and *E. tenella in vivo* occurs at the villus epithelium (Lillehoj and Trout, 1993; Shirley *et al.*, 2005). Before initiating endogenous development however, these species travel to the intestinal crypts where they invade another cell of the intestinal epithelium (Jeurissen *et al.*, 1996; Shirley *et al.*, 2005). Although the mechanism by which sporozoites travel from the villi to the crypts is not fully understood, it is believed to occur through the interaction with intestinal lymphocytes (Lawn and Rose, 1982). There are some species of *Eimeria* whose life cycles involve migration out of the gastrointestinal tract. Sporozoites of the rabbit coccidium, *E. stiedae*, migrate from the duodenum to the liver (Pakandl, 2009). Two species of *Eimeria* which infect cranes, *E. reichenowi* and *E. gruis*, produce a disease known as disseminated visceral coccidiosis, where zoites can be found in diverse organs such as the lungs, liver and heart (Novilla and Carpenter, 2004). The specific mechanisms involved in this type of migration are not fully understood; traffic *via* portal vein as well as the lymphatic system was an initial hypothesis (Fitzgerald, 1970); alternatively, spread throughout the host organism until settle in the liver has been suggested (Durr, 1972).

Schizogony

The next phase of development in *Eimeria* species consists of two to five rounds of asexual replication known as schizogony, where nuclear divisions and cellular expansion occurs to produce a multinuclear schizont [Figs 2(4), 2(5) and 6(2)]. The number of rounds of schizogony, the number of nuclear divisions and the specific site of development are specific characteristic to each species of *Eimeria* parasite. *Eimeria tenella* has three generations of schizogony, all located in the caecal crypts, whereas *E. maxima* has four-to-five generations mostly located in the villi of the small intestine (McDonald and Rose, 1987; Dubey and Jenkins, 2018). In the early stages of schizogony (up to 35 h post invasion) proliferation of the parasite endoplasmic reticulum occurs and nuclear divisions result in multiple granular nuclei, each enclosed by a perforated double membrane. During these divisions, intranuclear spindles, centrocones and centrioles can all be seen (Pacheco *et al.*, 1975). Centrioles of *Eimeria* have a $9 + 1$ singlet microtubule pattern, as opposed to the nine triplet symmetry found in mammalian cells (Dubremetz and Elsner, 1979). As the replicating schizont forms, the parasite significantly increases in size and may occupy up to half of the host cell content (McLaren, 1969).

After nuclear division, individual merozoites begin to develop in the form of protrusions of the schizont cytoplasm that develop a conoid at the apex [Fig. 6(3)]. Each merozoite then elongates and receives a single nucleus from the schizont. The mitochondria and apicoplasts (non-photosynthetic plastid organelle) of zoites of *Eimeria* and other coccidian species contain nucleic acid genomes that must be replicated and segregated into each of the forming merozoites. Apicoplast replication in *Eimeria* species occurs *via* a different mechanism to that in *T. gondii* tachyzoites, which divide by endodyogeny (rather than schizogony) with apicoplasts dividing in close association with centrosomes and in synchrony with nuclear division (Striepen *et al.*, 2000). In *E. tenella*, over 95% of sporozoites contain a single apicoplast with up to 5% having two or three of them, whereas to 20% of merozoites have multiple apicoplasts (Ferguson *et al.*, 2007). After zoite invasion, *E. tenella* apicoplasts enlarge to form pleomorphic-shaped

structures that divide several times during the proliferative phase of schizogony. This is not associated with centrosomes and occurs independently of nuclear division by an unknown mechanism (Ferguson *et al.*, 2007). Correct segregation of daughter mitochondria during schizogony is also poorly understood. Once schizonts are fully formed, the posterior poles of merozoites undergo constriction by cytoskeletal rings until they separate from what remains of the schizont, known as the residual body (McLaren, 1969; Pacheco *et al.*, 1975). The events that occur during the second round of schizogony in *E. tenella* and *E. necatrix* are particularly interesting since parasite infection causes the host cells to detach from adjacent cells and migrate deeper into the underlying tissue (Stockdale and Fernando, 1975; Fernando *et al.*, 1983; del Cacho *et al.*, 2004). In *E. tenella*, the second generation schizonts are larger than first generation schizonts, and the third generation schizonts are significantly smaller, containing less than 16 merozoites per structure (McLaren and Paget, 1968; Lee and Long, 1972; McDonald and Rose, 1987).

Merozoites share many characteristics and features of the sporozoite (Fig. 5C). They are bound by a triple bilayer pellicle (plasma membrane and double-layered IMC) and contain a posteriorly located nucleus of similar appearance to that of the sporozoite (McLaren and Paget, 1968). Amylopectin granules, endoplasmic reticulum, Golgi and an apical complex are also present; however they do not contain RBs, just a refractile dot. Second generation merozoites have been reported to have more micronemes but fewer rhoptries than their sporozoite counterparts (McLaren and Paget, 1968; Pacheco *et al.*, 1975). A couple of 'extra structures' have also been reported in newly formed merozoites, namely rod-shaped mitochondria and a vacuole with an electron dense outer membrane (McLaren, 1969).

Gametogony and fertilization

Upon re-invasion, the final generation of merozoites initiates a single round of sexual replication, however due to limitations of *in vitro* development little is known about this stage in *Eimeria* species. Despite some studies reporting a complete reproduction of the life cycle *in vitro*, this system is still very deficient, meaning that investigation of the sexual stages requires the use of a host animal (Hermosilla *et al.*, 2002). Microscopy of tissue infected *in vivo*, revealed that *Eimeria* species develop two sexually dimorphic stages; the macrogamete and the microgamete (Walker *et al.*, 2013). Macrogametes are large cells, measuring over 9 μm by 16 μm, and contain numerous polysaccharide storage granules for providing nutrients to the developing oocyst (McLaren, 1969). They also contain multiple structures known as wall forming bodies and veil forming bodies which are important for production of the oocyst wall (Ferguson *et al.*, 2003). Microgametes are considerably smaller, around 0.5 μm by 5 μm, and possess two flagella which enhance their motility needed for reaching and fertilizing a macrogamete (Madden and Vetterling, 1977). The formation of microgametes occurs in a similar way to the formation of merozoites by schizogony. Multiple nuclear divisions are performed followed by differentiation of mature flagellated microgametes, roughly 100 from each initial cell. This is markedly different from the process of microgamete formation that occurs in the haemosporines (including *Plasmodium* species) where microgametogenesis occurs as a result of chemical cues from the insect vector and involves extremely rapid exflagellation (the whole process taking around 8–15 min) (Sinden and Croll, 1975; Billker *et al.*, 1998).

Fertilization of the macrogamete by a microgamete results in the formation of the zygote, which is encased by the forming oocyst wall prior to excretion with the feces [Fig. 2(6) and 2(7)] (Jeurissen *et al.*, 1996; Shirley *et al.*, 2005). Ferguson *et al.* examined the ultrastructure of *T. gondii* microgametes (Ferguson *et al.*, 1974): these contain a dense nucleus, a single mitochondrion, two flagella which arise from basal bodies located within the cytoplasm and an osmophilic plate under the plasma membrane at the anterior of the cell. It was also observed that the number of microgametes produced were much lower than expected, meaning that there is no room for wastage if every macrogamete is to be fertilized (Ferguson, 2002). Two hypotheses have been presented to explain this phenomenon: (1) viable oocysts can be produced in absence of fertilization, and (2) the adaptive sex ratio theory, where, due to the high likelihood of inbreeding, selection pressure leads to production of only the minimal number of microgametes required for fertilization of the macrogametes (Ferguson, 2002; West *et al.*, 2003).

Parasite manipulation of the host cell

With most of their development occurring within a vacuole in the cytoplasm of another cell, coccidian parasites have an intimate relationship with the host (Jeurissen *et al.*, 1996). *Eimeria tenella* is incapable of *de novo* synthesis of purines and therefore must salvage these in a pre-formed state, relying on the host metabolism for this compound (LaFon and Nelson, 1985). With *E. bovis*, infection has been shown to significantly modify the host cell, altering gene expression relating to cell metabolism, cell structure, protein synthesis and gene transcription, suggesting that the parasite is able to manipulate the host cell in multiple ways that are advantageous to its survival (Lutz *et al.*, 2011). In *T. gondii* there is evidence that the parasite uses multiple mechanisms to intercept the normal apoptotic pathways of the host cell and thereby prevent destruction of its immediate environment. One such mechanism used by *T. gondii* involves activation of the transcription factor nuclear factor κB (NF-κB). Results of immune-histochemical staining of parasitized chicken tissue suggest that this pathway is also utilized by species of *Eimeria* in avoiding host cell apoptosis (del Cacho *et al.*, 2004). The intracellular development of *E. bovis* is particularly slow, compared to other *Eimeria* species, taking around 2 weeks to complete the first round of replication, forming exceptionally large schizonts (300 μm) known as macromeronts (Lutz *et al.*, 2011). In order to maintain host cell viability for this time, despite the pressures of parasitism, it seems especially likely that *E. bovis* is able to disrupt the apoptotic pathways of the host; indeed in cultured cells heavily infected by *E. bovis*, it is ultimately the uninfected cells which are seen to die off, whilst the infected cells survive (Lang *et al.*, 2009). These infected cells were shown to have increased expression of anti-apoptotic factors such as cellular Flice inhibitory protein (c-FLIP) and cellular inhibition of apoptosis protein 1 (c-IAP1).

Discussion

Parasites of the *Eimeria* genus are highly complex organisms, containing numerous structures and exhibiting complex life cycle and processes, some of which are markedly different from higher eukaryotes. Regardless of their high impact and wide prevalence, there are many mechanisms and morphological features that remain completely uncharacterized in *Eimeria* spp. Although *T. gondii* is an invaluable resource for inferring information, in particular regarding early endogenous replication, where proteins from specific secretory organelles (micronemes, rhoptries and dense granules) are essential for attachment, invasion, formation and modification of the intracellular parasitophorous vacuole and modulation of host cell pathways; it is a different organism and the pathogenesis of the two parasites differs slightly. Whereas the mechanisms of invasion are similar, the

intracellular development is significantly different. *Eimeria* has an acute, monoxenous life cycle, with no parasite stages persist within host tissue. In the other hand, *T. gondii* has a heteroxenous life cycle with acute and chronic phases; when ingested by intermediate hosts, parasites transform into tachyzoites that are found transiently in many tissues before they migrate to neural and muscle tissues, where they convert to the tissue cyst bradyzoites that remain in host tissues for life.

Therefore, potential targets and strategies for the control of toxoplasmosis would differ to those to control coccidiosis. For example, previous work on genomics and proteomics of *E. tenella* (Oakes *et al.*, 2013; Reid *et al.*, 2014) has shown an excellent conservation between *Eimeria* and *Toxoplasma* micronemes and rhoptry neck proteins involved in the first stages of endogenous development. However, there is much more limited conservation of rhoptry bulb proteins. In addition, there is very little conservation of genes encoding dense granules proteins (GRA). The different aim of the parasitophorous vacuole created by sporozoites (residing for short term leading to sexual reproduction) *vs* tachyzoites (establishing a chronic infection, eventually) could be one of the answers to this variable composition in ROPs and GRAs. This together with the lack of electron microscope evidence raise the significant question of whether *Eimeria* parasites contain organelles equivalent to dense granules of *Toxoplasma* and other cyst-forming coccidian. It is also interesting that the largest organelle of the parasite cell (RBs) with potential compounds that could serve as a target for disease control, still have an undetermined function.

Since the boom of molecular biology towards the end of the 20th century, there has been a decrease in microscopy-led biological research. However, both light and electron microscopes are invaluable tools for studying organisms such as *Eimeria*, which have limited *in vitro* systems and molecular tools for gene editing. For many biological structures, morphology is closely linked to function. Microscopy-derived data can therefore help answer questions about the function of subcellular structures and even individual proteins. In organisms with extensive genetic toolkits (such as *T. gondii*. or *Trypanosoma* spp.) microscopy can still be used to determine protein location and function, following the use of fluorescent tagging and protein synthesis disruption. As development of genetic techniques for species of *Eimeria* progresses, it is likely that these techniques will also play an important part in unravelling the biology of this species. Additionally, advances in cell culture systems and in genetic modification tools for *Eimeria* species (e.g. CRISPR/Cas9) could play an important role in answering some of the many questions regarding the functions and properties of eimerian subcellular structures and organelles such as the RBs, secretory organelles, apicoplast and conoid, each of which could potentially contain molecules that for targeting by novel drugs due to their absence in the cells of higher eukaryote host species.

Conclusions/future directions

In this paper, we have reviewed what is known about the life cycle and developmental stages of members of the *Eimeria* genus. An overarching aim in apicomplexan disease research is the production of affordable and sustainable vaccines and there is therefore a wealth of studies focused on the identification and testing of possible immunoprotective antigens. However, the identification of new candidates will not be possible without a complete understanding of eimerian biology. Investment in *in vitro* systems to get further in the parasite life cycle and testing alternative compound to control the disease are paramount, together with the development of new molecular tools for gene edition in *Eimeria* spp.

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Ethical standards. Not applicable.

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