REVIEW ARTICLE

Sex and *Eimeria*: a molecular perspective

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SUMMARY

Eimeria is a common genus of apicomplexan parasites that infect diverse vertebrates, most notably poultry, causing serious disease and economic loss. Like all apicomplexans, eimerians have a complex life cycle characterized by asexual divisions that amplify the parasite population in preparation for sexual reproduction. This can be divided into three events: gametocytogenesis, producing gametocytes from merozoites; gametogenesis, producing microgametes and macrogametes from gametocytes; and fertilization of macrogametes by microgametes, producing diploid zygotes with ensuing meiosis completing the sexual phase. Sexual development in *Eimeria* depends on the differential expression of stage-specific genes, rather than presence or absence of sex chromosomes. Thus, it involves the generation of specific structures and, implicitly, storage of proteins and regulation of protein expression in macrogametes, in preparation for fertilization. In *Eimeria*, the formation of a unique, resilient structure, the oocyst wall, is essential for completion of the sexual phase and parasite transmission. In this review, we piece together the molecular events that underpin sexual reproduction in *Eimeria* and use additional details from analogous events in *Plasmodium* to fill current knowledge gaps. The mechanisms governing sexual stage formation and subsequent fertilization may represent targets for counteracting parasite transmission.

Key words: *Eimeria*, Coccidia, Apicomplexa, macrogamete, microgamete, oocyst.

INTRODUCTION

Eimeria is a genus of at least 1200 species of obligate intracellular protozoan parasites within, in hierarchical order, the Coccidiasina, Conoidasida, Apicomplexa and Alveolata (Adl *et al*. 2005). The genus is relatively closely related to other coccidian genera such as *Cyclospora, Toxoplasma, Neospora, Hammondia, Isospora* and *Sarcocystis*, less closely to *Cryptosporidium*, and more distantly to haemosporidians such as *Plasmodium* (Adl *et al*. 2005).

Eimeria infect fish, reptiles, birds and mammals, with most species being highly host-specific. Most are parasites of the intestine but some do infect other gastric organs. The *Eimeria* that infect chickens are the best known and studied because they cause losses as high as US$2 billion per year to poultry industries around the world (Wallach *et al*. 2008); species that infect chickens include *Eimeria tenella, Eimeria maxima, Eimeria acervulina, Eimeria brunetti, Eimeria mitis, Eimeria necatrix* and *Eimeria praecox*. Genome sequencing projects are underway for *E. tenella* (www.genedb.org/Homepage/Etenella) and *E. maxima* (Blake *et al*. 2012).

The life cycles of poultry *Eimeria* are, arguably, the most straightforward of any apicomplexan parasites. Transmission is via the fecal–oral route and infection begins with the ingestion of sporulated oocysts, each of which contains eight haploid sporozoites, housed within four sporocysts (Fig. 1). These sporozoites invade intestinal cells, immediately enter sequential phases of asexual reproduction (referred to as merogony or schizogony) resulting in the production of merozoites, which are also haploid and reinvade the intestinal epithelia. The main purpose of this asexual phase of reproduction seems to be to amplify the sheer numbers of merozoites within the host in preparation for sexual reproduction, which is an important feature of all apicomplexan life cycles. Furthermore, as these parasites are haploid for much of their life cycle, there is an important implication for their genetics, namely that the progeny of asexual proliferation will be clonal. In addition, their life cycle is such that there is a real possibility of self-fertilization, which will also result in clonal offspring.

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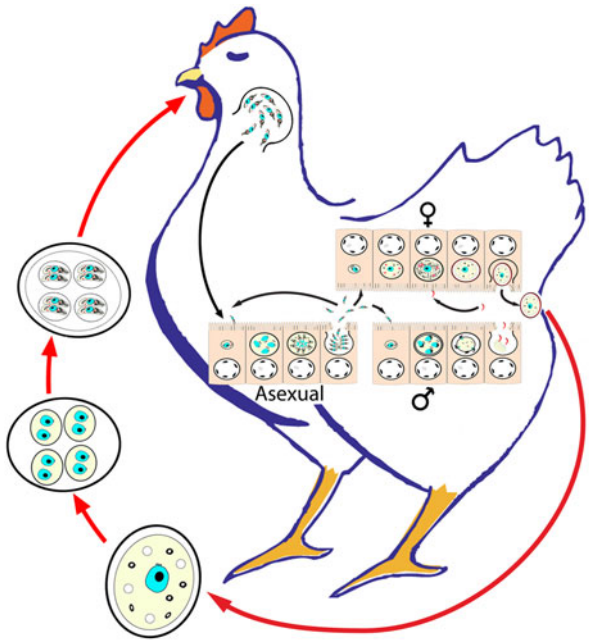


Fig. 1. Diagram illustrating the life cycle of *Eimeria* species in the chicken. Asexual development (merogony) of the poultry *Eimeria* species occurs in the intestine, giving rise to microgametes and macrogametes, the sexual stages of the life cycle. Fertilization of macrogametes by the flagellated, motile microgametes results in diploid zygotes, around which form oocyst walls. Unsporulated oocysts are excreted in the feces and sporulate under appropriate conditions of temperature and humidity. These sporulated oocysts, each containing eight infectious sporozoites, within four sporocysts, contaminate the external environment and infect new hosts when ingested with food or water. (Image supplied by D. J. P. Ferguson.)

That sex exists in *Eimeria*, and apicomplexan parasites, generally, is beyond dispute. Direct evidence for it includes the development of sexually dimorphic stages, genetic recombination events including meiosis, and the production of spores/cysts as part of the sexual phase of the life cycle. Morphological analyses of the dimorphic sexual stages and evidence of fertilization events have been documented to varying degrees for *Eimeria* (Klimes *et al*. 1972; Long, 1972; Fernando, 1973; Joyner and Norton, 1975; Speer and Danforth, 1976; Ferguson *et al*. 1977; Madden and Vetterling, 1977; Shirley, 1978; Pittilo and Ball, 1979, 1980; Ball *et al*. 1981; Elwasila, 1983; Sutton *et al*. 1986; Laxer *et al*. 1987; Shirley and Harvey, 1996; Blake *et al*. 2004), *Toxoplasma gondii* (Pelster and Piekarski, 1971, 1972; Ferguson *et al*. 1975; Grigg *et al*. 2001; Speer and Dubey, 2005; Sibley and Ajioka, 2008), *Cryptosporidium* (Beier and Sidorenko, 1990; Cheadle *et al*. 1999; Feng *et al*. 2002; Hijjawi *et al*. 2004), *Theileria* (Mehlhorn *et al*. 1975; Mehlhorn and Schein, 1976, 1977; Katzer *et al*. 2006) and *Plasmodium* (see, for example, MacCallum, 1897; Walliker *et al*. 1971, 1975, 1987; Sinden and Croll, 1975; Sinden *et al*. 1976; Ranford-Cartwright *et al*. 1991; Day *et al*. 1992; Babiker *et al*. 1994; Ramasamy *et al*. 1997; Conway *et al*. 1999; Moreira *et al*. 2004; Hastings and Watkins, 2005).

The apicomplexan sexual phase can be broadly divided into three main events: gametocytogenesis, producing gametocytes from merozoites; gametogenesis, producing haploid microgametes and macrogametes from gametocytes; and fertilization of macrogametes by microgametes, producing diploid zygotes with ensuing meiosis marking the end of the sexual phase (Sinden, 1983; Smith *et al*. 2002). However, variations in apicomplexan sexual reproduction patterns are apparent. Thus, in *Eimeria* and other Coccidia, there is no transfer of gametocytes between hosts; gametocytogenesis and gametogenesis represent a continuous process within one host cell. In contrast, in *Plasmodium*, gametocytogenesis and gametogenesis are distinct. In *Plasmodium* gametocytogenesis, merozoites produce gametocytes whose development is 'frozen', preventing gamete formation within mammalian hosts; gametogenesis only occurs in insects, producing haploid microgametes and macrogametes from gametocytes. An additional difference is that, in *Eimeria* and other Coccidia, there is a single, synchronized phase of gametocyte production whilst in *Plasmodium* there can be multiple, staggered phases of gametocyte formation, the result being that gametocytes can be observed circulating in the blood for many months, presumably to ensure increased opportunities for infecting insect vectors.

The genetic determination of the sexually dichotomous microgametes and macrogametes in *Eimeria* is distinct from the development of male and female gametes in, for example, mammals, in which it is the presence or absence of a single gene, on the Y chromosome, that dictates gender assignment in diploid males and females, respectively (Tilmann and Capel, 2002). Thus, the production of either of the dimorphic haploid mammalian gametes, the sperm or ovum, is restricted to the sexually mature male or female, respectively. In contrast, *Eimeria*, *Toxoplasma* and *Plasmodium* are known to develop into either micro- or macrogametocytes from cloned lines of haploid merozoites (reviewed by Cornelissen, 1988). Hence, unlike mammalian gender types, micro- and macrogametes of any clonal strain of an apicomplexan species are genetically identical, yet still develop into morphologically distinct cells (Ferguson, 2002). Thus, apicomplexan sexual differentiation is a purely phenotypic phenomenon. This indicates that sexual differentiation and development is dependent on the differential expression of sexual stage-specific genes, rather than presence or absence of sex chromosomes.

In this review, we aim to piece together the molecular events that underpin sexual reproduction

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in *Eimeria* and use any additional detail that can be gleaned from analogous events in *Plasmodium* to speculatively fill current gaps in our knowledge. Such information is intrinsically fascinating from a biological standpoint and, potentially, extremely valuable in the design of novel control strategies.

GAMETOCYTOGENESIS

In *Eimeria*, a set number of rounds of asexual reproduction (merogony) precedes gametocytogenesis (McDonald and Rose, 1987). The exact number of rounds of merogony that takes place before gametocytogenesis varies for different species (Klimes *et al*. 1972) but the norm may be three, at least in poultry *Eimeria* (McDonald and Rose, 1987). The commitment of final-generation merozoites to sexual differentiation is often considered to be genetically programmed and not reliant on environmental cues (Smith *et al*. 2002). This is less tightly controlled in *T. gondii* in which a mixture of asexual and sexual stages is the norm. There is also at least one exception amongst *Eimeria* species, namely *Eimeria vermiformis* in immunodeficient mice, in which the parasite continues into additional rounds of asexual reproduction in the absence of immune pressure from the host (Rose, 1987).

The morphologically distinct micro- and macrogametocytes are generally regarded as the sexual stages in *Eimeria* (and other Apicomplexa) but it can be inferred that sex determination occurs in the generation of merozoites immediately preceding gametocytogenesis. Thus, despite lack of sexual dimorphism, the final-generation merozoites must also be considered sexually committed (reviewed by Cornelissen, 1988). Klimes *et al*. (1972) concluded that third-generation *E. tenella* merozoites can be differentiated by periodic acid Schiff's (PAS) staining, with PAS-positive merozoites developing into PAS-positive macrogametocytes whereas PAS-negative merozoites develop into PAS-negative microgametocytes (though this has proved difficult to confirm; D. J. P. Ferguson, unpublished observations). It is possible, therefore, that sexual stage genes may be expressed in merozoites, well before the biological development of gametocytes. The identity of these genes is unknown in *Eimeria* but some clues may be found in studies on *Plasmodium falciparum*. Although gametocytogenesis takes only a few hours in *Eimeria*, it is a complex, time-consuming process in *P. falciparum*, taking 9–12 days, and involving five stages of morphogenesis (see Dixon *et al*. 2012). This extended period of gametocytogenesis has been a boon for the study of this process and, as a result, far more information has been gathered for *P. falciparum* than for any other apicomplexan. However, any extrapolations from *Plasmodium* to *Eimeria*, although potentially very useful, must be done with caution.

Analogous to *Eimeria*, schizonts of *P. falciparum*, in the generation preceding gametocytogenesis, are committed to producing daughter cells of a single gender only (Silvestrini *et al*. 2000; Smith *et al*. 2000). There is, likewise, some controversy regarding the role of parasite genetics *vs* environmental cues in *Plasmodium* gametocytogenesis within the mammalian host (Alano, 2007), with observations that the rate of asexual multiplication (Smalley *et al*. 1981) and drug treatments (Buckling *et al*. 1999*a*, *b*), amongst a wide variety of stressors (Baker, 2010), can dictate the onset of gametogenesis. Nevertheless, there is also clearly a parasite genetic component governing gametocytogenesis in *Plasmodium* (Day *et al*. 1993; Kuehn and Pradel, 2010; Sinden *et al*. 2012).

Not surprisingly, given their functional and morphological differences from asexual parasites, the gametocytes of malaria parasites possess unique molecular signatures (reviewed expertly by Sinden, 2009; Baker, 2010). Transcriptomic analyses have detected specific upregulation of expression of over 200 genes during gametocyte development (Silvestrini *et al*. 2005). Similarly, *P. falciparum* DNA microarrays have been used to observe expression patterns of gametocyte stages both in comparison to asexual stages (Le Roch *et al*. 2003) and within different developmental stages of the gametocyte (Young *et al*. 2005), identifying clusters of at least 218 and 246 genes, respectively, expressed in gametocytes only. What regulates the expression of such clusters is, as yet, not known but a role for the evolutionarily conserved, eukaryotic translational repressor, Puf2, is conceivable since it has been implicated in gametocytogenesis in *P. falciparum* (Miao *et al*. 2010), though a similar role was not confirmed in *Plasmodium berghei* (Hughes *et al*. 2010).

At the protein level, pioneering analyses (Lasonder *et al*. 2002) documented over 900 proteins in gametocytes, 315 of which were found exclusively in gametocytes. Subsequently, in what is proving to be a landmark study, Khan *et al*. (2005) were able to separate highly pure micro- and macrogametocytes prior to proteomic analysis. Six hundred and fifty proteins were identified in the microgametocyte samples, with 236 (36%) of these determined to be exclusive to this parasite stage, while 101 of the 541 proteins (19%) in the macrogametocyte samples showed stage-exclusive expression. Furthermore, 69 proteins were present in the pure samples of both micro- and macrogametocytes but not in asexual parasite samples. Moreover, aside from the high percentage of hypothetical proteins identified in the microgametocyte- (68%) and macrogametocyte-exclusive (54%) proteomes, the respective proteomes mirror the known biology of these distinct parasite stages (Khan *et al*. 2005). For example, macrogametocytes contain more ribosomal and mitochondrial proteins, in accordance with the higher abundance

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of ribosomes observed in this cell type (Janse and Waters, 2004). In contrast, microgametocytes contain more axoneme-related and DNA replication proteins (Janse and Waters, 2004) and, again, this is reflected in their biology, specifically, the generation of flagella and the genome replication for a possible eight motile gametes. Proteomic analyses of purified microgametes (Sinden *et al*. 2010; Wass *et al*. 2012) confirmed these observations, identifying several dynein components (dyneins are conserved flagellar proteins; Sinden *et al*. 2010) and the armadillo repeat protein, PF16, which was demonstrated to be a key flagellar structural element of microgametes (Straschil *et al*. 2010). Given the conserved nature of many of these flagellar proteins, it is reasonable to expect that similar proteins will be uncovered in coccidian parasites; indeed, the genomes for both *E. tenella* and *T. gondii* contain sequences corresponding to PF16 (Briggs *et al*. 2004).

GAMETOGENESIS

Gametogenesis – the formation of microgametes and macrogametes – has been well studied in *Eimeria* (Klimes *et al*. 1972; Fernando, 1973; Speer and Danforth, 1976; Ferguson *et al*. 1977, 2003; Madden and Vetterling, 1977; Pittilo and Ball, 1979, 1980; Ball *et al*. 1981; Elwasila, 1983; Laxer *et al*. 1987) and it is recognized that these processes are similar in *T. gondii* (Ferguson *et al*. 1974, 1975; Ferguson and Dubremetz, 2007). However, the molecular basis for gametogenesis in *Eimeria* is not well understood; some clues may, again, be available from studies on malaria parasites.

Microgametogenesis

Microgametogenesis (Figs 2 and 3) in *Eimeria* (and *T. gondii*) takes place within a parasitophorous vacuole inside host gut epithelial cells and can be observed as early as 130 h post infection for *E. maxima* (Ball *et al*. 1981) or 120 h post infection for *E. tenella* (Laxer *et al*. 1987). The process of microgamete formation involves a growth phase, in which there is repeated nuclear division, followed by a differentiation phase in which the nuclei move to the gametocyte periphery (Fig. 2C). The two centrioles become the basal bodies from which the axonemes develop, resulting in flagellar growth into the parasitophorous vacuole (Fig. 2D and F). The axonemes of *Eimeria* (and *T. gondii*) have the typical 9+2 microtubular structure also characteristic of *Plasmodium* microgametes (Sinden *et al*. 1976) (Fig. 2A and B; Fig. 3B and C). The emergence of the developed flagella generally precedes that of the microgamete body in *E. maxima* (Ball *et al*. 1981), *Eimeria magna* (Speer and Danforth, 1976), *E. brunetti* (Ferguson *et al*. 1977) and *E. tenella* (Madden and Vetterling, 1977). This is followed by budding of the basal bodies, the electron-dense portion of the nucleus and an associated mitochondrion to form the microgamete (Fig. 2D). The mature microgametes are released into the parasitophorous vacuole, and consist of a nucleus, a mitochondrion, a number of longitudinally running microtubules, and two or three flagella (Scholtyseck *et al*. 1972; Fernando, 1973; Ferguson *et al*. 1977; Madden and Vetterling, 1977; Elwasila, 1983) (Figs 2E and 3A). The final number of microgametes formed from a single microgametocyte varies depending on the species, ranging from 20–30 for *E. acervulina* to 100 or more for *E. maxima* (Scholtyseck *et al*. 1972; West *et al*. 2000). A similar process operates in *T. gondii* (Fig. 2D and F, Fig. 3D and E), though each microgametocyte only produces 15–30 biflagellated microgametes (Ferguson and Dubremetz, 2007).

Despite the wealth of ultrastructural data on microgametogenesis in *Eimeria*, little is known about the molecular mechanisms that might dictate this development. However, it is known that the protein, MORN1, is associated with a ring-like structure from which microgametes bud from microgametocytes in both *Eimeria* and *T. gondii* (Ferguson *et al*. 2008) (Fig. 4C). There is also genomic evidence that *E. tenella* and *T. gondii* have intraflagellar transport proteins that are absent in *Plasmodium* but which are associated with axoneme/flagellar formation (Briggs *et al*. 2004). Nevertheless, we can again speculate that similarities between *Plasmodium* and *Eimeria* may be found in other aspects of microgamete development, particularly in the molecular mechanisms relating to the signal cascades that accompany transformation of gametocytes into microgametes, the egress of microgametes from host cells and fertilization.

The development of *Plasmodium* microgametocytes into microgametes is the result of a sophisticated molecular response of the parasite to its changing environment as it transits from mammalian to insect host. One of the earliest apparent responses is activation of the phosphoinositol pathway and release of calcium from cytoplasmic storage (Martin *et al*. 1994; Raabe *et al*. 2011); this activates various protein kinases (Billker *et al*. 2004; Khan *et al*. 2005; Rangarajan *et al*. 2005; Reininger *et al*. 2005, 2009; Tewari *et al*. 2005; Ishino *et al*. 2006; Siden-Kiamos *et al*. 2006; Dorin-Semblat *et al*. 2007; McRobert *et al*. 2008; Ranjan *et al*. 2009). These kinases regulate everything from parasite egress to DNA replication to nuclear division to flagella assembly (Sinden, 2009). Another kinase, the serine/arginine-rich protein kinase, is essential specifically for microgamete development; this kinase is highly conserved in eukaryotes, contributing to alternative splicing of mRNA and regulating mitosis, which may be underscoring its importance in microgamete development (Tewari *et al*. 2010). There are

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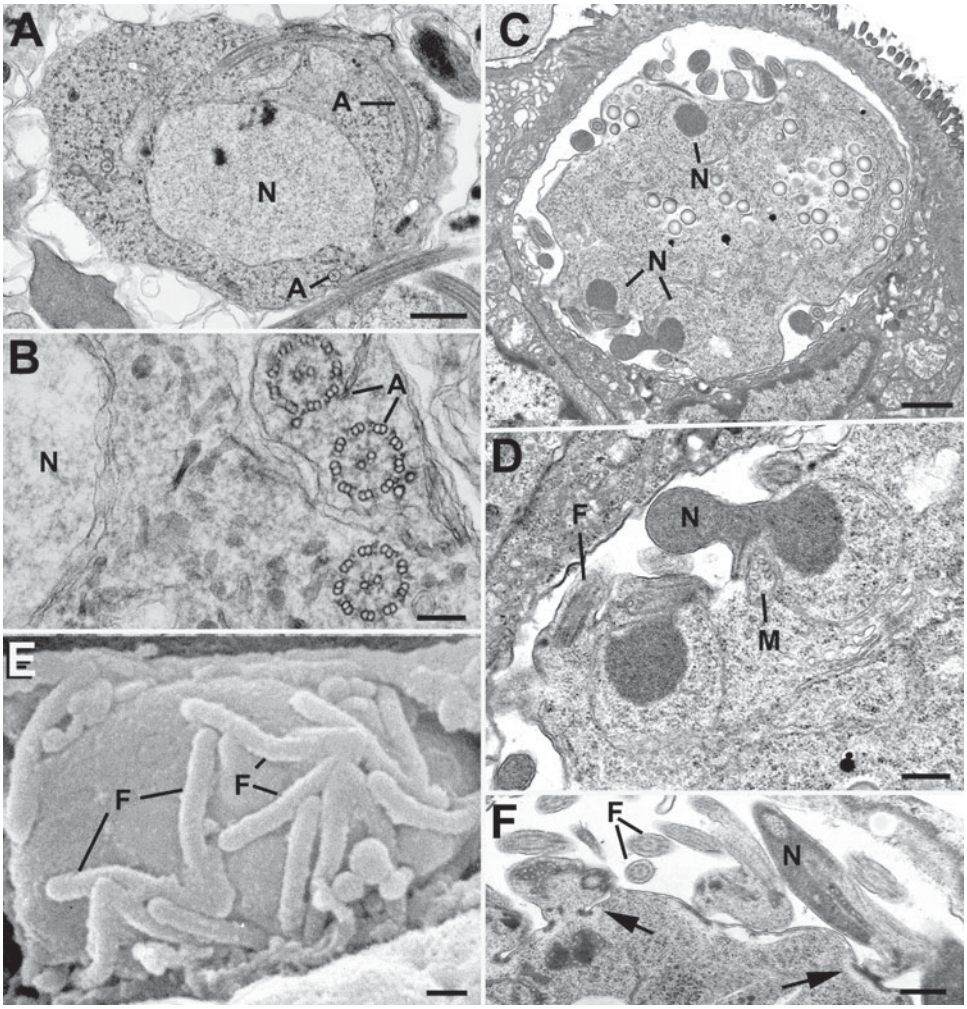


Fig. 2. Electron micrographs of microgametocytes and developing microgametes of *Plasmodium berghei* (A, B) *Eimeria tenella* (C, E) and *Toxoplasma gondii* (D, F). (A) Mid stage *P. berghei* microgametocyte showing the large nucleus (N) and a number of axonemes (A) located within the cytoplasm. Bar is 1 μm; (B) Detail of the *P. berghei* microgametocyte cytoplasm showing axonemes (A) with the classical 9 + 2 arrangement of microtubules. N – nucleus. Bar is 100 nm; (C). Mid-stage *E. tenella* microgametocyte showing a number of nuclei (N) located around the periphery. Bar is 1 μm; (D) Detail showing the electron dense portion of the nucleus (N) with associated mitochondrion (M) and flagella (F) protruding from the *T. gondii* microgametocyte surface into the parasitophorous vacuole. Bar is 200 nm; (E) Scanning electron micrograph of the surface of an *E. tenella* microgametocyte showing early stages of microgamete formation each with the two developing flagella (F). Bar is 100 nm; (F) Section through the periphery of a late-stage *T. gondii* microgametocyte showing microgametes within the parasitophorous vacuole but still attached by their anterior end to the gametocyte (arrows). N – nucleus, F – flagellum. Bar is 200 nm. (Images supplied by D. J. P. Ferguson.)

orthologues of several of these kinases in *T. gondii* and *Cryptosporidium parvum* (Billker *et al*. 2009) and it seems unthinkable that orthologues will not be found in *Eimeria* in due course. Similarly, conserved cell cycle proteins are crucial for microgamete formation in *P. berghei* (Guttery *et al*. 2012*a*) and their involvement in coccidian microgametogenesis might also be anticipated. Likewise, coccidian microgametes can reasonably be expected to contain the evolutionarily conserved histone chaperone, FACT-L, a protein identified by proteomic analysis (Khan *et al*. 2005) and subsequently demonstrated to be important for microgamete development (Laurentino *et al*. 2011). Finally, transcripts of several proteases are expressed during *Plasmodium* gametogenesis (reviewed by Kuehn and Pradel, 2010) and two independent studies (Torres *et al*. 2005; Rupp *et al*. 2008) have shown that treatment of *Plasmodium* gametocytes with cysteine/serine protease inhibitors can affect microgamete formation; *E. tenella* possesses a number of protease genes, the expression of which is upregulated or specific for the sexual stages (Katrib *et al*. 2012).

Macrogametogenesis

Macrogametogenesis in *Eimeria* occurs in a parasitophorous vacuole within the same type of host cell as the other stages. It occurs at specific post-infection time-points, which may vary depending on the

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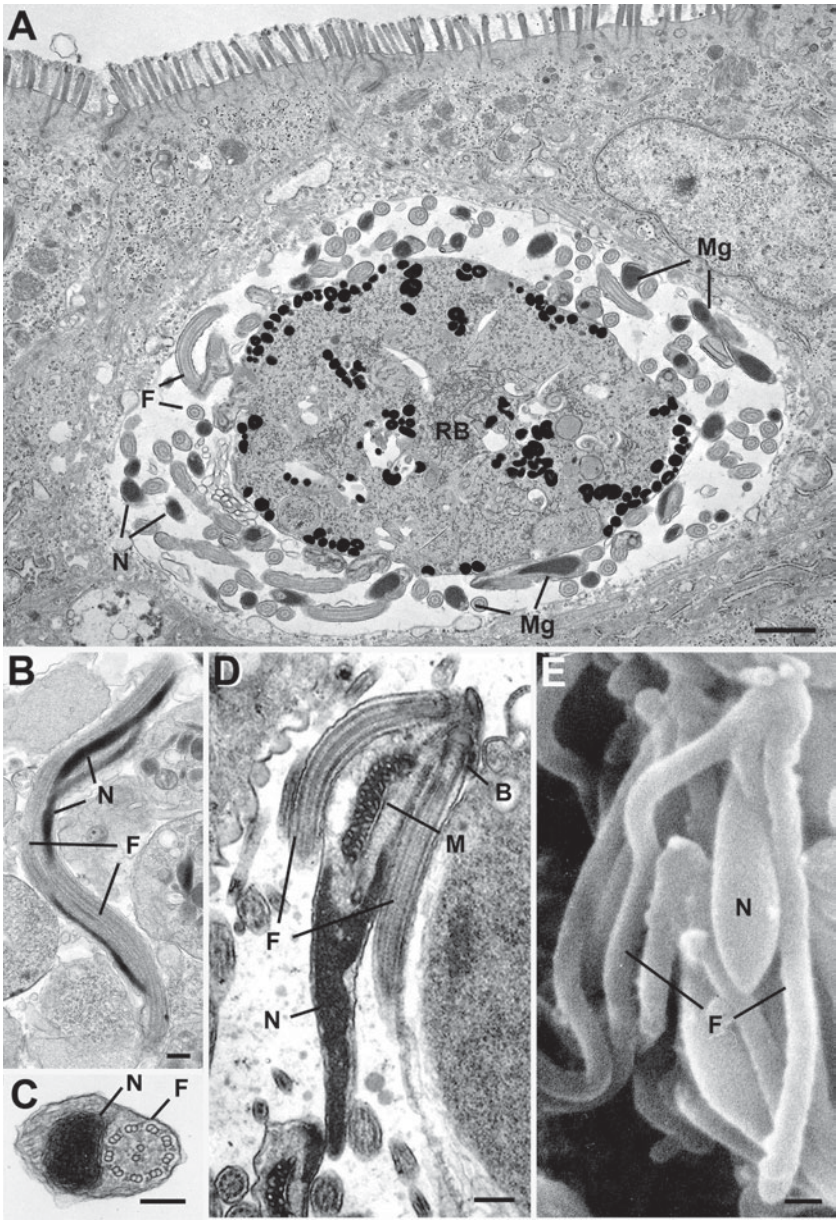


Fig. 3. Electron micrographs of mature microgametes of *Eimeria tenella* (A), *Plasmodium berghei* (B, C) and *Toxoplasma gondii* (D, E). (A) Low power micrograph showing a mature *E. tenella* microgametocyte surrounded by fully formed microgametes (Mg) within a parasitophorous vacuole in the apical cytoplasm of a gut epithelial cell. F – flagellum, N – nucleus, RB –residual body. Bar is 1 μm; (B, C) Longitudinal (B) and cross (C) section through a *P. berghei* microgamete showing the undulating flagellum (F) and the electron dense nucleus (N) enclosed by the plasmalemma. Bar is 100 nm; (D) Longitudinal section through a *T. gondii* microgamete showing the electron-dense nucleus (N) anterior to which is the mitochondrion (M) and the basal bodies (B) giving rise to two flagella (F). Bar is 100 nm; (E) Scanning electron micrograph of a mature *T. gondii* microgamete showing the body of the microgamete containing the nucleus (N) and the two long posteriorly running flagella (F). Bar is 100 nm. (Images supplied by D. J. P. Ferguson.)

species but, importantly, coincides with microgamete formation. *Eimeria tenella* macrogametogenesis can be observed as early as 120 h post infection (Klimes *et al*. 1972) while, in *E. maxima*, the same process can be observed as early as 130 h post infection.

Development of the macrogametes is characterized, most notably, by the synthesis of a repertoire of unique organelles – the veil-forming bodies (VFB), the wall-forming bodies, type 1 (WFB1) and type 2 (WFB2) – as well as storehouses in the form of polysaccharide granules and lipid droplets (see, e.g. Pittilo and Ball, 1979; Ferguson *et al*. 2003; reviewed by Belli *et al*. 2006). The VFB and WFB2 start to form before the WFB1. In chicken *Eimeria*, the WFB1 are osmiophilic (i.e. possibly containing lipid and protein) and are larger than the WFB2 (Pittilo and Ball, 1979; Ferguson *et al*. 2003) (Fig. 4A and B, Fig. 5C and D). However, in certain other *Eimeria*

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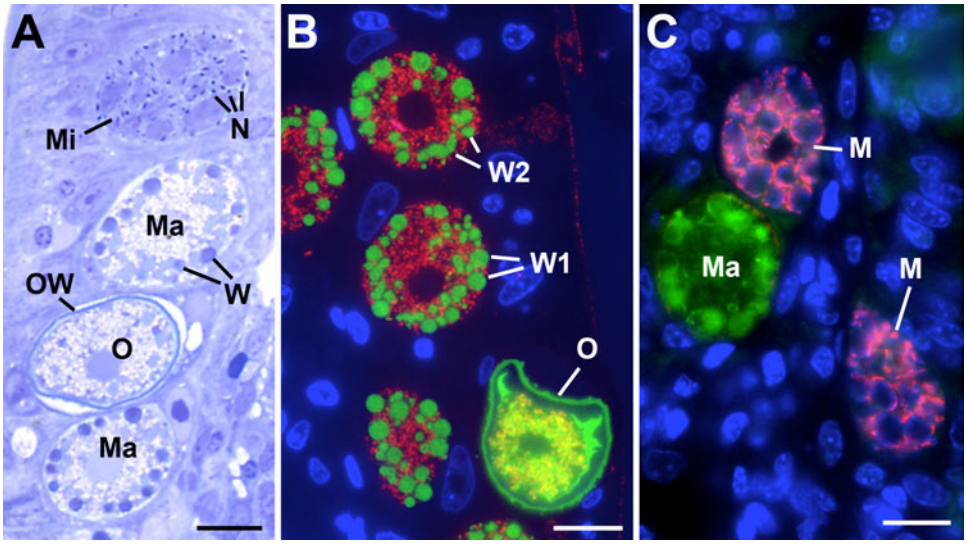


Fig. 4. Light micrographs of plastic (A, B) and wax (C) sections stained with Azure A (A) or by immunofluorescence (B, C) of gut tissue infected with *Eimeria maxima*. (A) Part of a villus showing a mid stage microgametocyte (Mi) with numerous peripherally located dark blue nuclei (N), mature macrogamete (Ma) with large wall forming bodies (W) and a developing oocyst (O) with formation of the oocyst wall (OW). Bar is 10 μm; (B) Similar section to A showing a number of macrogametes characterized by the positive staining (green) of the WFB1 and 2 (W1, W2) labelled with antibodies to affinity purified gametocyte antigens (see Ferguson *et al*. 2003) and a developing oocyst (O) characterized by the positive staining of the developing oocyst wall. Bar is 10 μm; (C) Similar section to A showing a macrogamete (Ma) identified by positive staining (green) with antibodies to affinity purified gametocyte antigens (see Ferguson *et al*. 2003), while two mid-stage microgametocytes (M) show positive staining (red) with anti-MORN1. Bar is 10 μm. (Images supplied by D. J. P. Ferguson.)

species and in *T. gondii*, the WFB1 are smaller than the WFB2 (Ferguson *et al*. 1974; Scholtyseck *et al*. 1971) (Fig. 5E and F). This is in marked contrast to *Plasmodium* in which there are no VFB, WFB, polysaccharide granules or lipid droplets (Sinden, 1982) (Fig. 5A and B).

The VFB are released during macrogamete formation and, following presumptive fertilization, the WFB1 and WFB2 ultimately form the outer and inner layers of the oocyst wall, respectively (discussed below). A limited number of proteins have been identified during eimerian macrogamete development, almost all of which are stored in wall-forming bodies and are, ultimately, incorporated into the developing oocyst wall (discussed below). Additionally, eimerian macrogametes carry the non-nuclear genome of the apicoplast, which exhibits maternal inheritance (Ferguson *et al*. 2007). However, information on other macrogamete-specific proteins that might be involved in other aspects of sexual stage biology is lacking and, again, clues may be sought from malaria parasites.

Plasmodium macrogametocytes respond to their uptake by mosquitoes in an almost identical manner to, and using similar molecular machinery as, microgametocytes, at least initially. They revert to a rounded morphology and egress from the red blood cell within a few minutes of arrival in their insect host's midgut (Sinden, 2009). Electron-dense, gametocyte-specific, osmiophilic bodies (Fig. 5A and B) (Sinden, 1982) are mobilized and release Pfg377 (Alano *et al*. 1995; Severini *et al*. 1999) and MDV-1/PEG-3 (Furuya *et al*. 2005; Silvestrini *et al*. 2005; Lanfrancotti *et al*. 2007; Lal *et al*. 2009), proteins that, along with GEST (Talman *et al*. 2011), play crucial roles in egress (de Koning-Ward *et al*. 2008; Ponzi *et al*. 2009).

Whilst microgametes are little more than a vehicle to deliver DNA, macrogametes are a molecular storehouse for the next generation of parasites. In the Coccidia, examples of this include polysaccharide granules and lipid droplets, which are abundant in macrogametes (Fig. 5C and E), and the storage, in the wall-forming bodies of coccidian parasites, of precursor proteins that are destined for processing and incorporation into the oocyst wall (see below and Belli *et al*. 2006 for a review). Another example is the translational repression that is initially evident during macrogametocytogenesis in malaria parasites and carried through the macrogamete until after fertilization. This phenomenon is mediated by DOZI (development of zygote inhibited), a member of the DDX6 family of RNA helicases, and CITH (a homologue of *Caenorhabditis elegans* CAR-1 and *Drosophila* Trailer Hitch), both of which are essential for zygote development (Mair *et al*. 2006, 2010). DOZI and CITH are core to the formation of *Plasmodium* P granules, within which around 20% (> 370) of macrogamete mRNA transcripts are stabilized, hidden from the cell's translational

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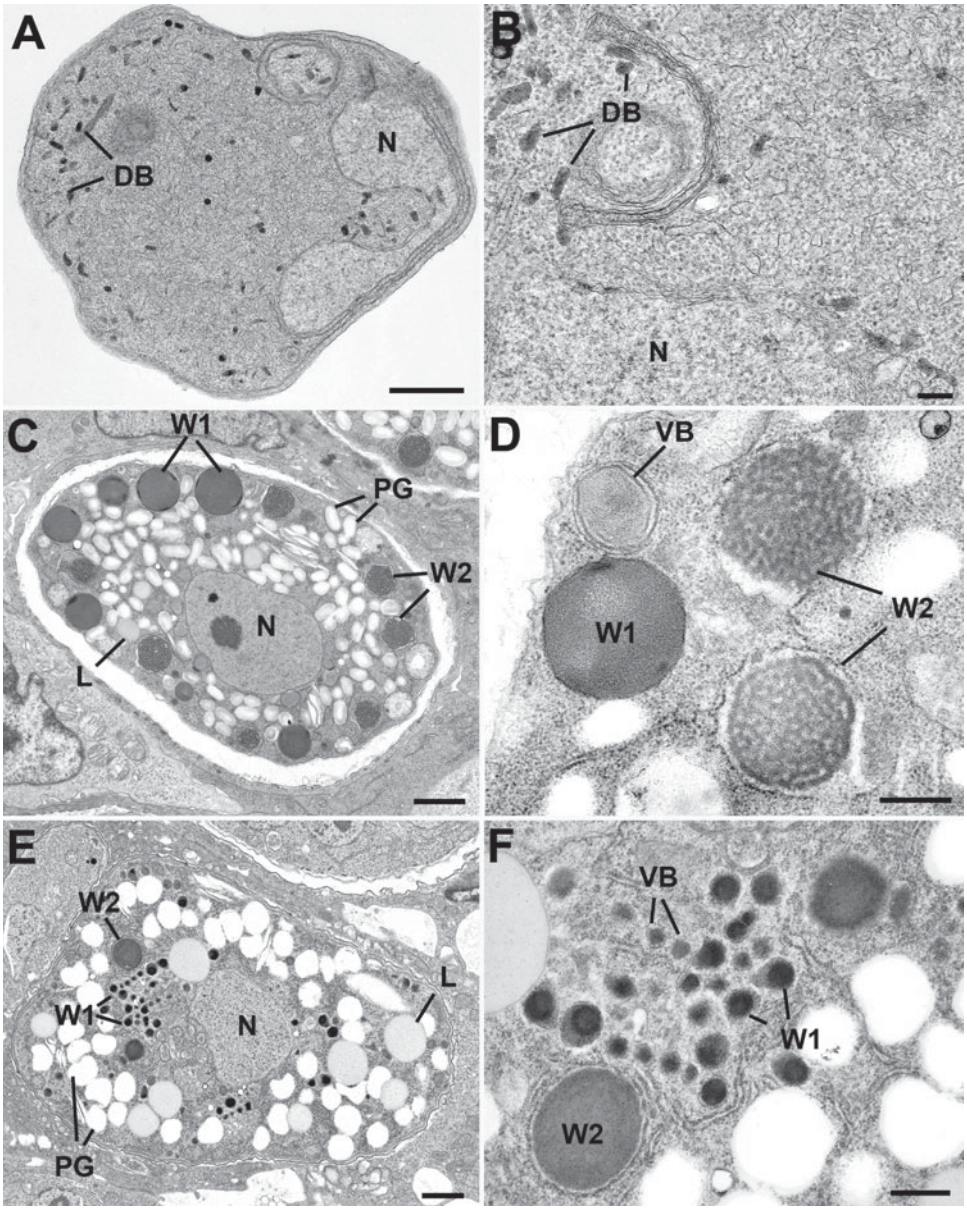


Fig. 5. Electron micrographs of macrogametes of *Plasmodium berghei* (A, B), *Eimeria tenella* (C, D) and *Toxoplasma gondii* (E, F). (A, B) Low power (A) and detail (B) of the macrogamete of *P. berghei* showing the nucleus (N) and the cytoplasm containing a few cigar-shaped dense bodies (DB). Bars are 1 μm and 100 nm, respectively; (C, D) Low power (C) and detail (D) of the macrogamete of *E. tenella* showing the nucleus (N) while the cytoplasm contains a number of veil-forming bodies (VB), large electron-dense WFB1 (W1) and a number of WFB2 (W2), which are located within membranes of rough endoplasmic reticulum, plus large numbers of polysaccharide granules (PG) and lipid droplets (L). Bars are 1 μm and 500 nm, respectively; (E, F) Low power (E) and detail (F) of the macrogamete of *T. gondii* showing the nucleus (N) while the cytoplasm contains a number of veil-forming bodies (VB) (identified by immunoelectron microscopy), small electron dense WFB1 (W1), and a few larger WFB2 (W2), plus large numbers of polysaccharide granules (PG) and lipid droplets (L). Bars are 1 μm and 200 nm, respectively. (Images supplied by D. J. P. Ferguson.)

machinery and, hence, stored until after fertilization (Mair *et al*. 2006, 2010). This process of translational repression is highly conserved in eukaryotic germline cells (Hughes *et al*. 2010) and, therefore, it may be anticipated that DOZI and CITH will be detected in the macrogametes of *Eimeria* in the not too distant future. However, it must be kept in mind that the needs of *Eimeria* and *Plasmodium* are very different. Within the Coccidia, there is a need to take up and store all the molecular requirements to fulfil subsequent development (sporulation), which occurs within the oocyst in the external environment and forms only a limited number of infectious sporozoites (eight in *Eimeria* and *Toxoplasma*), and also to maintain their viability for months. In contrast, the *Plasmodium* macrogamete, upon fertilization, converts into a motile ookinete before forming an oocyst at the basal lamina of the insect host's gut wall, where it obtains nutrients, undergoes marked growth and produces large numbers of sporozoites.

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FERTILIZATION AND MEIOSIS

The observation of fertilization *per se* has proved elusive in Apicomplexa such as *Toxoplasma* or *Eimeria* but it is known that zygotes form and develop into oocysts, which are excreted into the outside world and, within which, the sexual phase continues as meiosis, followed by mitosis, leading to the generation of haploid sporozoites over several days. It has, however, been postulated, due to the lack of solid empirical observations of fertilization, that the development of oocysts in Coccidia could take place without the necessity of fertilization, for instance, via parthenogenesis (Ferguson, 2002). However, in *Eimeria* there is some evidence that fertilization of macrogametes by microgametes is necessary for the production of a viable oocyst. First, monoclonal antibodies raised against purified *E. tenella* microgametes have been shown to reduce oocyst formation *in vitro* by greater than 50% (Laxer *et al*. 1987) and, second, a correlation was noted between reduced microgamete numbers (following prolonged culturing at sub-standard temperatures) and poor sporulation rates in oocysts (Long, 1972). Together, these findings suggest that, while some oocysts may still be produced and excreted in the absence of fertilization, they may ultimately fail to sporulate (i.e. produce sporozoites) and are, therefore, non-infective. Furthermore, although fertilization may be empirically presumptive in *Eimeria*, genetic recombination has been shown convincingly in *E. tenella* (Shirley and Harvey, 1996), *E. maxima* (Joyner and Norton, 1975), *E. acervulina* (Sutton *et al*. 1986) and *E. mitis* (Shirley, 1978), and also for *T. gondii* (Pfefferkorn and Pfefferkorn, 1980). Furthermore, genetic recombination has been shown to be a potential mechanism for the development of drug resistance in *E. maxima* (Blake *et al*. 2004).

Little is known of the physical process of fertilization in Coccidia with the exception of a few preliminary ultrastructural studies based on limited observations showing microgametes in contact with macrogametes (Scholtyseck *et al*. 1971; Madden and Vetterling, 1977; Elwasila, 1983) (Fig. 6A). It is not even clear when or where fertilization takes place, since microgametes have no cell-invading machinery, which could suggest it occurs extracellularly (in the gut lumen). However, oocyst wall formation is initiated while the macrogamete is still within the host cell (Fig. 4A and B, Fig. 6B). How the microgamete could penetrate the oocyst wall is unclear. Currently, no proteins or molecular events underpinning fertilization or meiosis in *Eimeria* have been discovered and, again, we must turn to *Plasmodium* for clues.

Fertilization in *Plasmodium*, in contrast to other Apicomplexa, is well documented (Kuehn and Pradel, 2010); indeed, it was observed over 100 years ago (MacCallum, 1897) and described in detail nearly 40 years ago (Sinden and Croll, 1975; Sinden *et al*. 1976). Interaction between microgametes and macrogametes in the lead-up to fertilization appears to be mediated by a number of surface proteins with adhesive properties (reviewed by Sinden, 2009; Baker, 2010). These include P45/48, P47 and P230, which are all members of a family of proteins characterized by the possession of 6-cysteine domains. P47 is macrogamete-specific (van Schaijk *et al*. 2006) whereas P45/48 and P230, though found in both micro- and macrogametes, seem to be more important in microgamete fertility (van Dijk *et al*. 2001; Sinden, 2009). These proteins may form surface complexes as a prelude to fertilization (Sinden, 2009). Another group of adhesive or attachment proteins is the CCP family (Simon *et al*. 2009), at least one of which is known to associate with P230 (Scholz *et al*. 2008), which is highly suggestive of an important role in microgamete to macrogamete binding (Baker, 2010). What role recently discovered, nanotube-like filaments (Kuehn and Pradel, 2010) play in bringing microgametes and macrogametes together, and whether this involves any of these adhesive proteins, remains to be determined. Whether similar processes operate in *Eimeria* (or other apicomplexans) is unknown but, hopefully, will be more readily analysable with the completion of *Eimeria* genome-sequencing projects.

In *Plasmodium*, once contact between micro- and macrogametes has been made, fusion of the gamete membranes proceeds quickly, mediated by HAP2/GCS1 (Hirai *et al*. 2008; Liu *et al*. 2008). Homologous sequences for *hap2/gcs1* are also found in the genomes of *T. gondii* and *E. tenella* (Sinden *et al*. 2012). Following membrane fusion, the microgamete moves into the cytoplasm of the macrogamete and, within hours, nuclear fusion occurs, resulting in a diploid zygote (Sinden *et al*. 1985). The zygote undergoes meiosis without cell division, becomes tetraploid and transforms into an ookinete over the next 24 h, thus marking the end of the sexual phase of the *Plasmodium* life cycle (Sinden *et al*. 1985; Janse *et al*. 1986). At a molecular level, the genes held dormant during gametocytogenesis in the mammalian host are now expressed, control being mediated by the combined efforts of transcription factors like AP2-O (Yuda *et al*. 2009), kinases such as Nek-1, Nek-4, PK7, GAK and CDPK1 (Reininger *et al*. 2005, 2009; Tewari *et al*. 2010; Sebastian *et al*. 2012), the translational repressors, DOZI and CITH (Mair *et al*. 2006, 2010), and the unique protein phosphatase with Kelch-like domains, PPKL (Guttery *et al*. 2012*b*), all of which are critical for zygote and/or ookinete development. At least some of these factors (e.g. Nek-1, Nek-4, DOZI) play important roles in, or set the stage for, meiosis (Reininger *et al*. 2005, 2009; Mair *et al*. 2010) and, given their evolutionarily conservative

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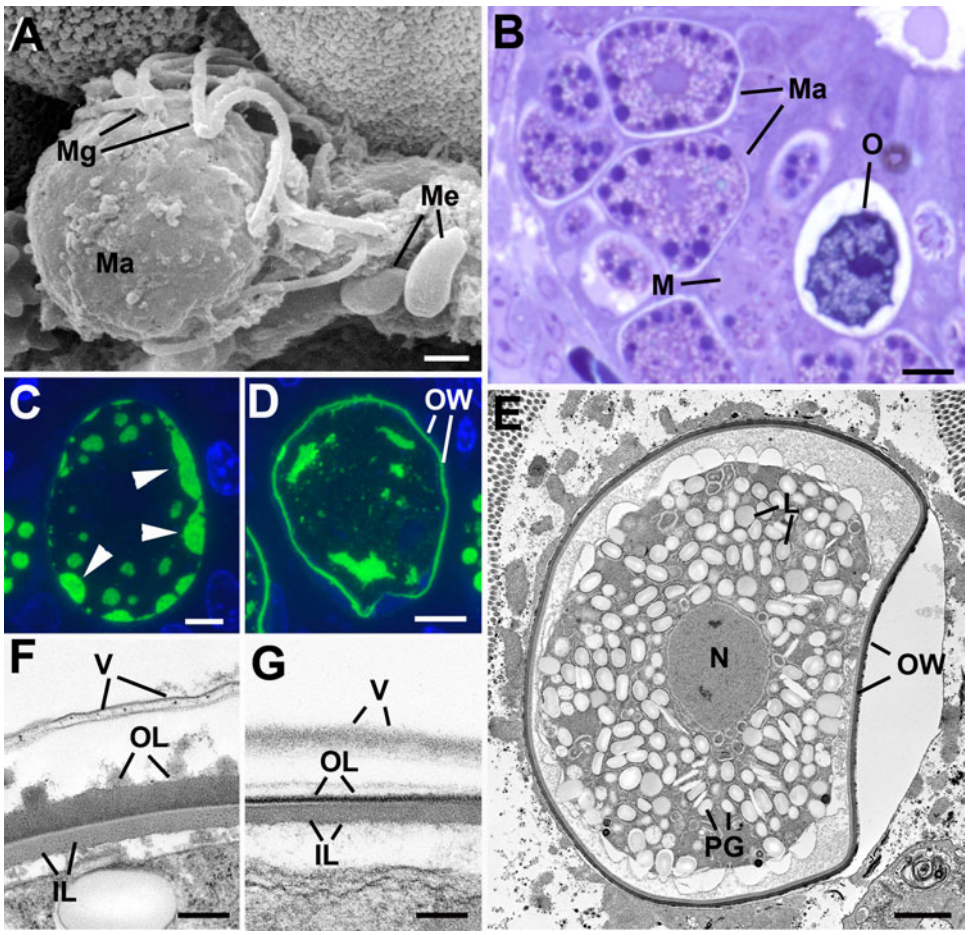


Fig. 6. Images of macrogamete and oocyst formation in *Toxoplasma gondii* (A, G), *Eimeria tenella* (B, E, F) and *Eimeria maxima* (C, D). (A) Scanning electron micrograph showing a number of microgametes (Mg) characterized by their flagella in contact with a macrogamete (Ma) that could represent a stage in fertilization in *T. gondii*. Me – merozoite. Bar is 1 μm; (B) Light micrograph of a section through a chicken caecum infected with *E. tenella* showing a number of mature macrogametes (Ma), a few microgametocytes (M) and a developing oocyst (O) that is still located within a host cell. Bar is 5 μm; (C) Immunofluorescent images showing *E. maxima* WFB1, positively stained (green) with antibodies to affinity purified gametocyte antigens (see Ferguson *et al*. 2003), coalescing at the surface to initiate oocyst wall formation (arrowheads) in *E. maxima*. Bar is 5 μm; (D) Immunofluorescent image showing a developing *E. maxima* oocyst with the wall (OW) positively stained (green) with antibodies to affinity purified gametocyte antigens (see Ferguson *et al*. 2003) in *E. maxima*. Bar is 5 μm; (E) Low-power transmission electron micrograph of a fully formed oocyst of *E. tenella* in the gut lumen, showing the oocyst wall (OW) enclosing a cytoplasmic mass that, having lost its WFBs, consists of a central nucleus (N) surrounded by polysaccharide granules (PG) and lipid droplets (L). Bar is 1 μm; (F) Detail of the periphery of the *E. tenella* oocyst in E showing the loose outer veil (V) and the wall consisting of the thick electron dense outer layer (OL) and the more electron lucent inner layer (IL), while the cytoplasmic mass is limited by the plasmalemma. Bar is 100 nm; (G) Detail of the periphery of an oocyst of *T. gondii* showing the loose veil (V) and the wall consisting of a thin electron dense outer layer (OL) and the more electron lucent inner layer (IL). Bar is 100 nm. (Images supplied by D. J. P. Ferguson.)

natures, might be anticipated to be important in zygote development in parasites like *Eimeria* and *Toxoplasma*.

The result of fertilization and meiosis in *Plasmodium* is the ookinete, which traverses the insect midgut epithelium and forms an oocyst between the basal lamina and the gut epithelium. However, unlike the oocysts of *Eimeria, Plasmodium* oocysts are not excreted and are not a site for any further sexual development. Rather, this oocyst stage remains in the insect, a marked growth phase ensues and a series of endomitotic divisions occurs; the resulting sporozoites burst from the oocyst and invade the salivary glands, ready for subsequent transmission to the mammalian host (Sinden, 2009). In contrast, for *Eimeria* and other Coccidia, the oocyst is the stage in which sexual reproduction is completed; meiosis proceeds, inside the protective oocyst wall, followed by mitosis to produce the infectious sporozoites. Thus, assembly of the oocyst wall is a critical part of sexual reproduction for coccidian parasites.

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OOCYST WALL DEVELOPMENT

The formation of an oocyst wall through the coalescence of veil- and wall-forming bodies in macrogametes is an event that is well documented, not only in various species of *Eimeria* (Scholtyseck *et al*. 1971; Mouafo *et al*. 2002; Ferguson *et al*. 2003; Belli *et al*. 2009), but also in other cyst-forming Apicomplexa such as *Sarcocystis* (Vetterling *et al*. 1973), *T. gondii* (Scholtyseck *et al*. 1971; Ferguson *et al*. 1975, 2000; Speer and Dubey, 2005) and *C. parvum* (Spano *et al*. 1997). Many of the components of the oocyst wall are produced and stored during macrogamete development; the similarity in macrogamete development in different cyst-forming Apicomplexa is clearly related to the conserved biology of this remarkable structure. The oocyst wall acts as a protective barrier that shields the parasite from harsh, external pressures faced during transmission between hosts, including environmental stress and chemical disinfection (Belli *et al*. 2006).

Ultrastructural morphology and biochemical composition

The oocyst wall of *Eimeria* is a bilayered structure and is composed of an outer layer and inner layer, which incorporate the contents of WFBs type 1 and 2, respectively (Stotish *et al*. 1978; Pittilo and Ball, 1980; Elwasila, 1984; Ferguson *et al*. 2003) (Figs 4B and 6C–F). Early studies indicated that the outer layer is made up of lipid material, whereas the inner layer is comprised mainly of disulphide-linked glycoprotein, evidenced mainly by the dissociation effect of thiol reagents (Stotish *et al*. 1978). Recent analyses of purified *E. maxima* and *E. tenella* unsporulated oocyst walls (containing both the inner and outer layer) demonstrated a protein content exceeding 90% in both instances (Mai *et al*. 2009). The *T. gondii* oocyst wall has also been described as bilayered, although, in contrast to *Eimeria* species, the electron-dense outer layer is thinner than the inner layer (Ferguson *et al*. 1975) (cf. Fig. 6F and G). The developing oocyst is enclosed by an outer veil, initially described for *T. gondii*, but also present in *Eimeria* species (Ferguson *et al*. 2000, 2003; Belli *et al*. 2009) (Fig. 6F and G). This veil is lost by the time that oocysts are excreted with the feces.

Molecular composition

A number of proteins, ranging in size from 8 to 33 kDa, found in purified wall samples of *E. maxima* oocysts, can be mapped to the amino acid sequence and/or have been shown to cross-react with antibodies raised against the macrogamete-specific proteins, GAM56 (Belli *et al*. 2003*a*) and GAM82 (Belli *et al*. 2003*b*), respectively. This suggests that gametocyte-specific proteins are processed into smaller peptides prior to their incorporation into the oocyst wall. Similar observations have been made for other *Eimeria* species (Mouafo *et al*. 2002; Krucken *et al*. 2008; Belli *et al*. 2009). It is not completely clear what mechanism is governing the processing of these gametocyte-specific proteins prior to their incorporation into the oocyst wall, although Katrib *et al*. (2012) have proposed that parasite-specific proteases are involved.

GAM56 and GAM82 contain one and two tyrosine-rich domains, respectively, in their amino acid sequences. Following the predicted processing of these proteins, fragments of GAM56 and GAM82 are proposed to cross-link, via the oxidative catalysis of dityrosine bond formation, to form an extracellular matrix around the developing zygote or oocyst (Belli *et al*. 2003*a*, 2006; Mai *et al*. 2011). The role of tyrosine-rich proteins in the development of extracellular matrices has also been documented for other parasites, including *Schistosoma mansoni* (Chen *et al*. 1992) and *Fasciola hepatica* (Waite and Rice-Ficht, 1987), specifically as major components of their eggshells. No direct homologues of GAM56 or GAM82 can be found in *T. gondii* but six tyrosine-rich proteins have been found in the oocysts of this parasite (Fritz *et al*. 2012*a*, *b*). No homologues exist in any of the *Plasmodium* genomes, probably reflecting the fact that malaria parasites do not produce an excreted oocyst or develop a hardened oocyst wall.

A second class of oocyst wall protein has also been identified in *E. tenella* and also appears to represent a WFB2-specific protein. This protein, GAM22, is a histidine- and proline-rich glycoprotein that has been detected in mature gametocytes (at 168 h post infection) and unsporulated oocysts (Krucken *et al*. 2008). It is expressed much later than GAM56 (Mouafo *et al*. 2002). Unlike GAM56, there is no indication that GAM22 is processed into smaller peptides prior to wall formation. However, as proposed by Krucken *et al*. (2008), it is tempting to speculate that it may play a role in stabilizing the oocyst wall via cross-links between histidine and catechols, as seen in insect cuticles.

We have also very recently identified (unpublished) a third class of oocyst wall protein in *E. tenella* and *E. maxima,* namely, homologues of the cysteine-rich *Cryptosporidium* oocyst wall proteins (COWPs). There are nine known COWPs in *Cryptosporidium*, all displaying varying degrees of conservation of Type I and Type II cysteine-rich repeats (Lally *et al*. 1992; Ranucci *et al*. 1993; Spano *et al*. 1997; Templeton *et al*. 2004). At least some of these appear to localize to the inner oocyst wall, the wall-forming bodies of macrogametes and to a single large vesicle within immature macrogametocytes, indicating this protein is potentially an early indicator of sexual stage development. Furthermore, semi-quantitative RT-PCR analyses revealed upregulated transcription

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of all nine genes in parasite stages associated with sexual stage development and oocyst formation in *C. parvum*, supporting the hypothesis that these COWP proteins are involved in the formation of the oocyst wall. The study by Templeton *et al.* (2004) also uncovered a homologue of COWP6 in the *T. gondii* genome sequence (referred to as TgOWP1) and, subsequently, seven further COWP homologues have been uncovered in *T. gondii* (Possenti *et al.* 2010). As is the case for tyrosine-rich proteins, no plasmodial homologues to COWP have been found.

Various authors (Lally *et al.* 1992; Ranucci *et al.* 1993; Spano *et al.* 1997; Templeton *et al.* 2004, Possenti *et al.* 2010) have speculated that disulphide bonding between cysteine residues of COWPs may play a role in stabilizing the structure of the oocyst wall. Interestingly, the cyst walls that form around the gamonts of another apicomplexan, *Gregarina niphandrodes* (Gregarinasina), also have a cysteine-rich protein that shows high similarity to COWP proteins (Omoto *et al.* 2004). Like *Cryptosporidium, Toxoplasma* and *Eimeria*, this organism produces a cyst that is excreted via the feces and transmitted to a new, susceptible host (a mealworm beetle). Even in more divergent organisms, like the cnidarian, *Hydra vulgaris*, the development of the cyst wall (in this case, the nematocyst wall) appears to represent a conserved mechanism involving the cross-linking of highly-repetitive, cysteine-rich proteins (Engel *et al.* 2002; Ozbek *et al.* 2004).

Carbohydrates may also play critical roles in oocyst wall construction. The primary enzyme in the glycosylation pathway, glucosamine : fructose-6-phosphate aminotransferase has been characterized in *E. tenella* and is expressed specifically in macrogametes, transcription occurring early in gametocyte development but protein expression being restricted to mature gametes prior to their conversion into oocysts (Walker *et al.* 2010). Genes coding for three other enzymes needed for *N*-galactosamine synthesis are also transcribed in the course of *E. tenella* macrogamete development and both GAM56 and GAM82 contain high levels of *N*-galactosamine (Walker *et al.* 2010). Thus, it is possible that O-linked glycosylation of these proteins plays a role in directing them to the developing oocyst wall at the appropriate time. Additionally, carbohydrates may be important oocyst wall constituents in their own right since β-1,3-Glucan has been proposed to form a structurally critical, trabecular scaffold in the inner layer of the oocyst wall of both *T. gondii* and *E. tenella* (Bushkin *et al.* 2012).

CONCLUSIONS AND PERSPECTIVES

Understanding of the mechanics of sexual reproduction and transmission of apicomplexan parasites has advanced significantly in recent years, proving to be more than just an esoteric pursuit; it has resulted in the commercialization of a transmission-blocking subunit vaccine against *Eimeria* species, preventing coccidiosis in flocks of poultry (Wallach *et al.* 2008). This vaccine contains a complex of immunogenic proteins, purified from the gametocytes of *E. maxima*, including GAM56 and GAM82 (Belli *et al.* 2002, 2003*b*), proteins that localize to the wall-forming bodies of the macrogamete, the fused wall-forming bodies in the developing zygote, and the inner layer of the bi-layered wall of the resulting oocyst (Ferguson *et al.* 2003). Vaccination with these proteins stimulates an immune response that is hypothesized to negate construction of the oocyst wall, a protective barrier necessary for the completion of the parasite's sexual phase, and for survival in the outside world prior to transmission to the next host (Sharman *et al.* 2010). This vaccine illustrates that transmission-blocking immunity can be implemented successfully to control apicomplexan parasites.

It is not far-fetched to believe that similar control can be achieved for other diseases caused by apicomplexans. Indeed, irrepressible momentum to develop and implement strategies to interrupt transmission of *Plasmodium*, as a critical element of a campaign to eradicate malaria, has shone an intense spotlight on the sexual phase of the apicomplexan life cycle (Alonso *et al.* 2011; Sinden *et al.* 2012). A transmission-blocking vaccine against *T. gondii*, in which case the sexual stage-specific feline host represents a bottleneck in the parasite's life cycle, is also an attractive target.

Garnering a thorough understanding of sexual stage biology in the Apicomplexa will be fundamental to designing and implementing any effective transmission-blocking strategy; the successful design of transmission-blocking vaccines that target the sexual stages of *Eimeria* species has only been possible due to decades of research dedicated to this biological process. And, even in the case of this (limited) success story, less than absolute inhibition of transmission is a feature of vaccination (Wallach *et al.* 1995, 2008). This may be adequate, even optimal, for protection against poultry coccidiosis in the integrated, self-contained environments used to raise commercial poultry (Wallach *et al.* 2008) but will likely be less satisfactory for similar strategies to prevent transmission of, for example, malaria parasites or *T. gondii*. Perhaps the answer is multipronged vaccines that target, simultaneously: (a) the amplification of merozoites and/or their transformation into gametocytes; (b) the flagella of the microgametes, halting their ability to move to macrogametocytes; (c) proteins (presumably on the surface) of microgametes and/or macrogametocytes that mediate binding of the two in preparation for fertilization; and (d) in the case of coccidian parasites at least, proteins that give the oocyst wall its form and function.

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Although analogous in part to *Eimeria* species, sexual stage development in other apicomplexans is nonetheless sufficiently divergent to warrant an entirely new assessment of possible transmission-blocking strategies. Historically, the analysis of apicomplexan sexual stages has proven a challenging task, due in no small part to the lack of reliable systems for culturing and purifying these specific cell types. In recent years, the limitations associated with analysing minute quantities of often impure parasite samples have become more surmountable, with advances in both the reliability and affordability of high-throughput transcriptome sequencing (Otto *et al*. 2010; Fritz *et al*. 2012*b*). Although transcriptomics is unlikely to hold all the answers to sexual stage development – selective translational repression in *Plasmodium* species, for example, confounds transcriptome analysis somewhat – it has already provided important insights into the mechanisms of gene regulation and associated sexual stage determination. Thus, the discovery and investigation of AP2-O transcription factors (Balaji *et al*. 2005; Yuda *et al*. 2009), as well as translation regulators like DOZI (Mair *et al*. 2006), CITH (Mair *et al*. 2010) and CDPK1 (Sebastian *et al*. 2012) has already begun to yield insights into their roles in apicomplexan parasite sexual stage biology. Targeting such mechanisms may well be an effective approach to parasite control in the future via interference with the spectacular transformations that occur during sexual differentiation and development in apicomplexan parasites.

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