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Opinion

# The Symbiotic Spectrum: Where Do the Gregarines Fit?

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**Gregarine apicomplexans are closely related to parasites such as *Plasmodium*, *Toxoplasma*, and *Cryptosporidium*, which are causing severe health and economic burdens. Colonizing only invertebrates and having no obvious medical relevance, they are mostly ignored in 'omics' studies, although gregarines are the most basal apicomplexans and therefore key players in the understanding of the evolution of parasitism in the Apicomplexa from free-living ancestors. They belong to the largest exclusively parasitic phylum, but is this perception actually true? The effects of gregarines on their hosts seem to cover the whole spectrum of symbiosis from mutualistic to parasitic. We suggest future research directions to understand the evolutionary role of gregarines, by elucidating their biology and interaction with their hosts and the hosts' microbiota.**

## Highlights

Parasites are generally considered to be harmful, but current debates and ongoing research showed that they can actually have beneficial effects on their hosts.

Members of the Apicomplexa are unique microbial eukaryotes comprised entirely of symbiotic organisms, making it an ideal group to study the evolution of parasitism.

Gregarines are highly diverse, residing in most invertebrates, but their pathogenicity and influence on their host's population ecology is still in question.

Gregarines form the base of apicomplexan phylogenies. They are considered the most ancestral of the apicomplexans.

The use of advanced methods to explore the gregarines' biology is still in its infancy. To elucidate the evolution of parasitism in the apicomplexans, a comprehensive understanding of the gregarines' biology, genetic make-up, host–parasite interactions and explorations of the hosts' holobiome is required.

## Parasitism in the Apicomplexa

The phylum Apicomplexa contains unicellular parasites (currently more than 6000 named species) and is well known for its notorious pathogens of humans and livestock, such as *Plasmodium* (causative agent of malaria; mainly infecting humans, humanoids), *Toxoplasma* (toxoplasmosis; humans, cats), *Eimeria* (eimeriosis; poultry, cattle, ruminants), *Theileria* (theileriosis; cattle), *Babesia* (babesiosis; cattle, humans); *Isospora* (isosporiasis; humans), *Cyclospora* (cyclosporiasis; dogs, humans), and *Cryptosporidium* (cryptosporidiosis; humans, most livestock). These pathogens are of great public health concern and economic relevance, and they affect millions of people each year [1]. They all have intracellular life stages with the exception of *Cryptosporidium* [2], which has intracellular and extracytoplasmic stages [3,4]. Apicomplexans infect both invertebrates and vertebrates and have complex life cycles that differ considerably between the abovementioned groups [5] (Figure 1). Most of these life cycles involve at least two host species (i.e., a **heteroxenous** life cycle) (see Glossary). The apicomplexan clade is referred to in publications and textbooks as the largest phylum of eukaryotes that consists of obligate parasitic (Box 1) species only; but is this assumption really true for all apicomplexan species?

### The Gregarines

Within the apicomplexans, gregarines are a unique subgroup that infects a wide range of freshwater, marine, and terrestrial invertebrates (almost exclusively). Different views concerning the taxonomy of the gregarines are emerging [6,7], but comprehensive evidence for a reliable overall taxonomic review is still missing. The latest review of eukaryotes still refers to the historic major groups Archigregarinorida, Eugregarinorida, and Neogregarinorida, mainly based on habitat, host range, and trophozoite morphology, to which is added the Cryptogregarinorida to accommodate *Cryptosporidium* [8] (Figure 1). Archigregarines are the most ancestral group, with a mix of ancestral and derived features, occurring in marine habitats only. Eugregarines can be found in marine, freshwater, and terrestrial habitats with large trophozoites that are morphologically different from the infective sporozoites. Neogregarines have reduced trophozoites and infect

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only terrestrial, primarily insect, hosts. Archigregarines infect the intestines, while eugregarines are found in the intestines, coeloms, and reproductive vesicles, and neogregarines infect mainly the host tissues [9–11]. Apart from the vertebrate-infecting Cryptogregarinorida, the other three groups can be found in most invertebrates that have been investigated so far, including commercially important species and genera such as *Apis mellifera* (honey bee), *Crassostrea* spp. (oysters), *Litopenaeus* spp. (shrimps) [12–14]. More than 95% of described species on earth are invertebrates and therefore potential, but yet to be investigated, hosts for gregarines. With the assumption that, for example, millions of arthropod species have not yet been described [15], the sheer number of potential gregarine species lies in the millions [16,17] as gregarines tend to be very host-specific (e.g., *Psychodiella* spp. and their respective sand fly hosts [18]; diverse eugregarine species in cockroaches [19]) or sometimes even host's life-stage-specific (different *Gregarina* species in life stages of the mealworm beetle [20]). The life cycles of gregarines differ significantly from most of the other apicomplexans as they generally utilize only one host organism (**monoxenous** life cycle), but some exceptions do exist (e.g., *Nematopsis* spp. infecting crustaceans using mollusks as intermediate hosts). *Nematopsis* is also the only genus that has been reported from frog tadpoles [21], which is currently the first and only case of a eugregarine infecting a vertebrate host. Sexual and asexual cycles are extracellular in the single-host organism, which could be any invertebrate, setting the gregarines apart from the apicomplexans infecting vertebrates (for review see [11]; Figure 2, Key Figure). Gregarines have been identified in most invertebrate taxa [11], but intriguingly, they are yet to be discovered in rotifers and nematodes. It is worth mentioning that gregarines do also play a part in hyperparasitism. Gregarines infecting marine invertebrates are hosts to about 30 different species of Microsporidia in seven genera [11]. In contrast, only a few species of hyperparasitic gregarines have been reported. For example, the marine eugregarine *Monocystella batis* has been described from a rhabdocoel turbellarian parasitizing the Crown of Thorns starfish [22], and the terrestrial eugregarine *Steinina ctenocephali* infects the cat flea [23].

## Glossary

**Bacteriome:** the combined genomes of all bacterial species that exist on or inside a living organism.
**Heteroxenous:** refers to the life cycle of a parasite that involves at least two different host species.
**Microbiome:** the combined genomes of all microbes [bacteria (bacteriome), eukaryotic microbes/protozoa (eukaryome), fungi (mycobiome) and viruses (virome)] that exist on or inside a living organism.
**Microbiota:** the microbial taxa that exist in a specific environment (e.g., the human gut).
**Monoxenous:** refers to the life cycle of a parasite that has only a single host.
**Mutualism:** interaction between two or more different organisms, where each of them benefits from this relationship.
**Parasitism:** a relationship between two different organisms in which one benefits at the expense of the other.
**Symbiosis:** interaction between two or more different organisms existing in close physical association.

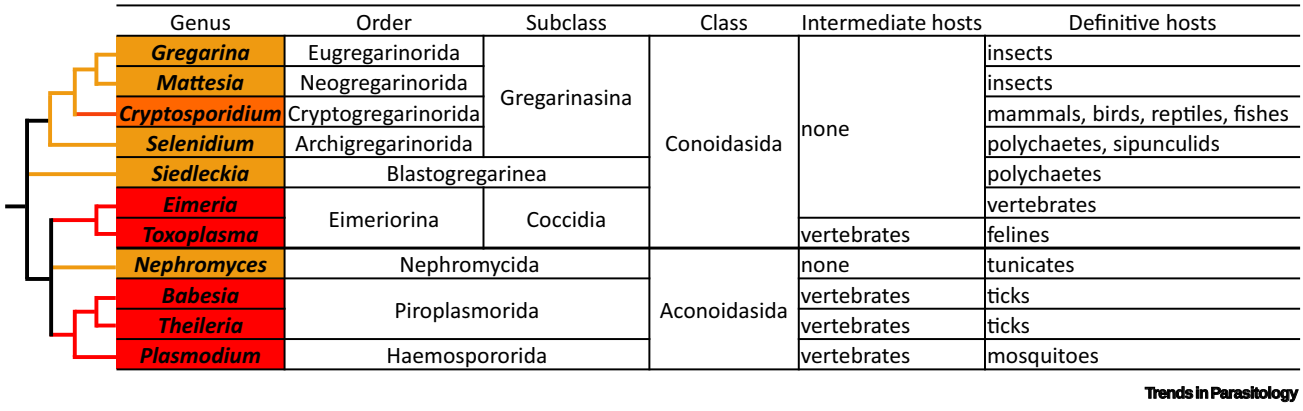


Figure 1. Simplified Phylogeny of Apicomplexans, Their Taxonomy, and Host Associations. The class Aconoidasida is comprised of the subclasses Haemosporida (e.g., *Plasmodium*), Piroplasmorida (e.g., *Babesia*, *Theileria*), and Nephromycida (e.g., *Nephromyces*). Three subclasses are also contained in the Conoidasida, the Coccidia (e.g., *Eimeria*, *Toxoplasma*), Gregarinasina (e.g., *Selenidium*, *Gregarina, Mattesia, Cryptosporidium*), and Blastogregarinea (e.g., *Siedleckia*). The Gregarinasina are the most basal in the apicomplexan tree. Most of the gregarines exemplified here by the genera *Gregarina, Selenidium*, and *Mattesia* are monoxenous, infecting only one invertebrate host. *Cryptosporidium* is also monoxenous, but it infects vertebrates. The position of the monoxenous, polychaete-infecting, Blastogregarinea (e.g., *Siedleckia*) is also basal, but not yet fully resolved. In the Coccidia, *Eimeria* is monoxenous, infecting vertebrates, while *Toxoplasma* is heteroxenous, using vertebrates as intermediate hosts and primarily felines as their final hosts. The Nephromycida are branching off at the base; they are monoxenous and infect only ascidians, predominantly of the genus *Molgula*. *Babesia* and *Theileria* represent the Piroplasmorida. They are both heteroxenous with different vertebrates as intermediate hosts, but both use ticks as their final hosts. The Haemosporida are heteroxenous with, for example, *Plasmodium* infecting humans as intermediate hosts and the mosquito as the final host. Colours in the schematic phylogenetic tree represent the spectrum from extracellular (light orange) to intracellular (red) parasitism.

### Box 1. What Is Parasitism?

With the ongoing debate of what parasitism actually is [56], and if all parasites are harmful, we could provide a plethora of definitions of parasitism that range from anything that lives in and nourishes from another organism [65] to all varieties of interspecific associations in a gradient of interdependence [66]. In a way, it depends on how specific terms to describe these interspecific relationships are used. On the one hand you can say that commensalism, mutualism, and parasitism are forms of symbiosis according to the definition of de Bary [67]. On the other hand parasitism could be used as the overall term describing an interspecific relationship without any implication of pathogenicity or benefit to one or both partners in that relationship [68], which would make the terms parasitism and symbiosis interchangeable. The determination of any specific cut-off points for the terms mutualism, commensalism, and parasitism in their classical sense is difficult, as their boundaries are plastic, within a gradient between the two most extreme forms [66]. We have used the term parasitism here as the parasite causing some form of harm to its host organism in order to tackle a major gap in the understanding of the evolution of parasitism in a major parasitic clade of public health concern. With the advancement of new techniques and the necessity of universal drug targets for devastating diseases, the opportunity arises now for scientists to join forces to address current shortcomings using our recommended approaches for a comprehensive understanding of the evolution of parasitism in the apicomplexans.

## Evidence of the Huge Diversity of Gregarines in Various Habitats

Recent metagenomic (metabarcoding) studies exploring the eukaryotic diversity in marine and terrestrial ecosystems have shown high diversity and dominance of apicomplexan parasites, specifically infecting invertebrates [24,25]. These observations support previous claims of high gregarine diversity, with millions of new species still to be discovered, making it one of the most diverse groups of eukaryotes [16,17]. For example, Mahe *et al*. [24] used a combination of metabarcoding and phylogeny-aware cleaning steps on samples from neotropical rainforests, demonstrating that gregarines were the predominant species (~80%) in soil. The high gregarine abundance was not a surprise, based on the extreme diversity of invertebrates (especially insects) in these areas. The authors suggested that gregarine infections could be a major limiting factor for host population growth that could otherwise become locally abundant/dominant [24], contributing to the high animal (invertebrate) diversity in these forests. While this is a valid assumption, it

## Key Figure

### The Symbiotic Spectrum towards Intracellular Parasitism in Apicomplexans

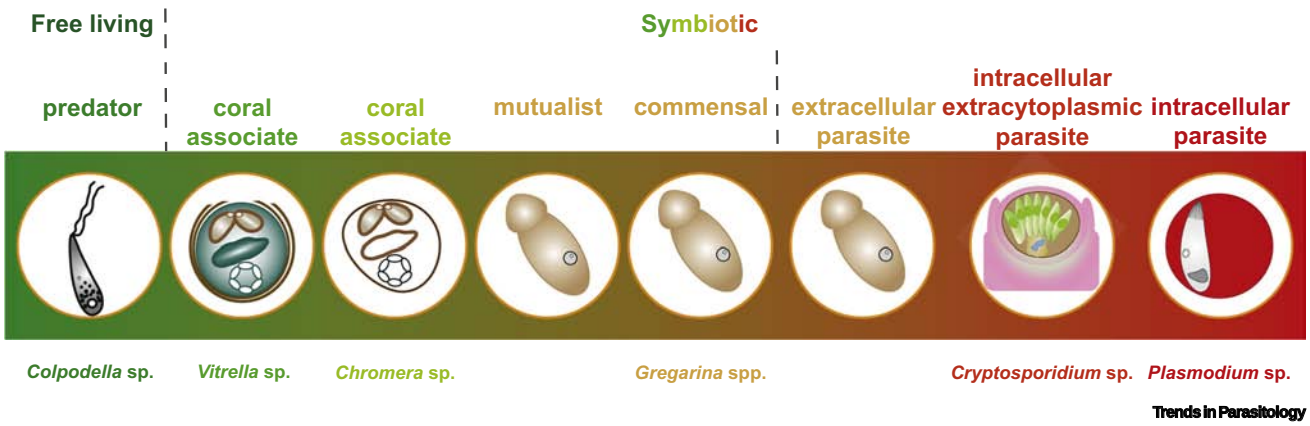


Figure 2. The cartoon depicts the major roles of organisms closely related to or within the apicomplexans on a spectrum from free-living to intracellular parasitism (green to red colouring of the spectrum). *Colpodella* is a free-living, heterotrophic species closely related to the apicomplexans and a voracious predator of other free-living single-celled eukaryotes. *Vitrella* is the closest known green, and *Chromera* is the closest known brown phototrophic relative to apicomplexans; both are associated with corals. As discussed, gregarines, exemplified here as *Gregarina* spp., cover a wide symbiotic spectrum, from mutualists, via commensals to parasites, and thus appear as intermediates in this spectrum. *Cryptosporidium*, now considered a gregarine, expands the spectrum as this organism is an intracellular and extracytoplasmic parasite, and lastly *Plasmodium* as the ultimate intracellular parasite.

does not fundamentally explain the high gregarine diversity in comparison to the number of arthropods. Due to the host-specificity of gregarines [19], it is expected that a different gregarine species resides within each arthropod species, and the huge diversity and number of gregarine species reflect the successful coevolution of gregarines and their hosts [26]. Thus, an alternative explanation of these results is that gregarines are not all parasitic, but that some are rather part of the host's natural flora (**microbiota**), not causing any severe harm, and potentially contributing to the host's fitness. Therefore, in our opinion, gregarines represent the whole spectrum of **symbiosis**, between the boundaries of **mutualism** and **parasitism**, representing the stepwise adaptations of the (intracellular) parasitic life style of the crown apicomplexans (Figure 2).

## What Is the Actual Life Strategy of Gregarines?

Gregarine infections are generally considered benign, unless the numbers of gregarines become large enough to impede passage of food through the host's gut [27,28]. Several reports have discussed potential negative effects of the presence of gregarines in host organisms, including reduced longevity and growth, increased mortality, and nutrient deficiency (see Table S1 in the supplemental information online). Most of these reports are focused on terrestrial invertebrates, while hardly anything is known about aquatic invertebrates except for some ascidians [28], bivalves such as oysters [29], or scallops [30] and crustaceans [14]. The effects range from high mortalities in ascidians [28] to negligible effects in bivalves [30]. *In vitro* experiments in insects have suggested that the number of gregarines can affect, for example, the overall flight performance and mating success of dragonflies [31], increase adult mortality and inhibit ovarian and fat body development in the western corn rootworm [32], mortality of immature sand fly stages and a negative effect on the survival of adult males and females [33]. Due to these negative effects on hosts that can be either disease vectors or pests, the potential of gregarines as biological control agents has been discussed [33,34], but their usefulness is often questioned by the authors. It has also been shown that gregarines can have positive effects (Table S1) on their host's development, fitness, and longevity [35]. Sumner [36] suggested that gregarines are even essential for the growth of mealworm beetle larvae. Bollatti and Ceballos [37] showed that pseudoscorpions survived longer when they carried high gregarine loads, compared to the group with low gregarine loads, but overall, they suggested a commensalistic relationship. According to a study by Kaunisto *et al*. [38], homozygous individuals of damselflies harboured more parasites, posing potentially strong selection pressure against inbreeding and homozygosity. There are many studies where no effect was reported. Klingenberg *et al*. [27] showed that even high loads of gregarines in the midguts of water striders had no effect on growth and development time, even under different rearing conditions. Another study found that gregarines had no effect on field crickets' weight, longevity, and fecundity, when fed *ad libitum* [39]. Significant effects were present, though, when the field crickets were reared under suboptimal diets [39]. The rare description of a gregarine in a tadpole did report that there were no signs of disease or the impairment of fitness or function due to the infection [21]. Gregarine infections do not necessarily occur in isolation. Fellous and Koella [40], for example, looked at the detrimental effect of a coinfection by two organisms (gregarine and microsporidium). They showed that it was not a fixed parameter, but dependent on the epidemiological context and the quality of the host's habitat.

Overall, we can claim that gregarines do cover the whole range of symbiotic relationships from mutualistic to parasitic within their hosts (Figure 2). Switching between the various forms of symbiotic relationships could be an option, depending on (extreme) environmental conditions or certain cues from the host organism. Little is known about these shifts, but there are examples of viruses and bacteria on an evolutionary level [41], as well as annelid and crayfish, where the host–symbiont cleaning relationship shifts due to a changing environment [42]. The question remains why a few gregarines cause harm to, or have positive effects on, their hosts

(see above), while (most) others seem to have none, and what does this mean from an evolutionary perspective?

## Gregarines Can Elevate Our Understanding of Parasitism and Its Evolution in the Apicomplexa

Recent phylogenetic studies have shown that gregarines, in particular the archigregarine group, have a distinctive position, forming the paraphyletic stem-group of gregarines and potentially all apicomplexans [9]. Still, most of the available phylogenies are based on 18S rRNA gene sequences only. While the genomes of some of the crown apicomplexans (e.g., *Plasmodium*, *Toxoplasma*), *Cryptosporidium*, as well as photosynthetic close relatives (e.g., *Chromera*, *Vitrella*) have been published [43], there is close to no genomic information available for the gregarine apicomplexans apart from a draft genome survey of the mosquito-inhabiting *Ascogregarina taiwanensis* [44], and an expressed sequence tag study of the gametocyst development in *Gregarina niphandrodes* from the mealworm beetle [45]. Consequently, most of the knowledge on cellular characteristics of gregarines stems from ultrastructural studies based on transmission and scanning electron microscopy [9,11]. So far, studies on gregarines are lacking aspects considering their genetic make-up to better understand their biology, fit and role within apicomplexans. The importance of these data has been shown in the newly discovered 'corallicolids', a basally branching apicomplexan lineage in corals [46]. The authors sequenced the apicoplast (the nonphotosynthetic plastid in the apicomplexans) genome and revealed the lack of genes encoding photosystem proteins, but the conservation of proteins for chlorophyll synthesis, making them evolutionary intermediates between their free-living and parasitic relatives [46]. There is evidence for an apicoplast on the cellular level for at least the archigregarines [47,48], but no data are available yet on the molecular level for comparison.

While this is another step in the understanding of the transition from phototrophy to parasitism (Figure 2), we need to survey the gregarines using a combination of 'omics' (genomics, transcriptomics, proteomics) approaches on species from all three major orders, the archi-, eu- and neogregarines to cover the whole spectrum of gregarine diversity in order to be able to trail the evolutionary steps in parasitism throughout the apicomplexans. It will be important to determine if, and potentially how, gregarines with different life strategies (mutualistic to parasitic) have adapted their biology according to their host-niche and perceived role. As *Cryptosporidium* has just recently been considered to belong to the gregarines taxonomically, it might be the 'go to' model system to adopt key approaches in future gregarine research (Box 2).

Exploring the host's microflora and the potential interactions between the **microbiome** and the gregarines will be another way to understand how they influence the host's gut and subsequently

### Box 2. *Cryptosporidium* Status Quo

While we are discussing whether gregarines are true parasites in its classical sense here, we have to mention *Cryptosporidium*, as this parasite has recently been suggested to be a gregarine [6,69]. The debate about the relationship between *Cryptosporidium* and the gregarines has not been settled yet, and the current phylogenetic placement of the Cryptosporidia has two important implications: (i) parasitism of vertebrates in the apicomplexans might have evolved twice (Figure 1), and (ii) if this proves to be true, *Cryptosporidium* could be used as a model system to elucidate the biology of gregarines. There have been rapid advancements in the methodological approaches [3] for the study of the parasites, including the development of an *in vitro* cell culturing system in cell lines [63] and organoids [70,71] along with the development of genetic tools such as CRISPR/Cas9 [72] and small interfering RNA (siRNA) [73]. In addition, there are several 'omics' methods that have been introduced including *in vitro* transcriptomics [74,75], proteomics [63,76], metabolomics [60], and state-of-the art microscopy techniques [63], including immunomicroscopy [63,77], to understand the invasion strategies and host–parasite interactions. These methods, though completely absent in the field of gregarine research at the moment, should be easily transferrable and would allow the exploration of the symbiotic state of gregarines.

the host's fitness. Many microbial eukaryotes, including *Candida albicans* [49], *Entamoeba histolytica* [50], *Giardia lamblia* [51], *Cryptosporidium* [52], *Tritrichomonas* [53], and even more extensively *Blastocystis* [54,55], have shown a distinct **bacteriome,** and potentially positive influence [53], when one of the aforementioned eukaryotes was present (for review see [56]). These eukaryotic microorganisms are known to cause diseases when the host becomes immunocompromised, or if there are significant alterations in the host's microbiome. It has been suggested that these 'pathogenic' microorganisms could play an important role in shaping the microflora, especially in the gut, and thus sustaining a host–microbiome balance [56]. For instance, in humans, it has been observed that healthier individuals often harbour greater microbial diversity [57,58], which was further correlated with the presence of microbial eukaryotic residents [55,56]. Consistent with these observations, a connection between the host microbiome and the prevalence of gregarines could also be possible. Invertebrates that harbour gregarine species could have a more diverse microbiome, which might be shaped by the presence of the gregarines, and future studies should explore this hypothesis. This would not only be a way to explain the presence of gregarines in almost every invertebrate species, but also their high infestation rates in some hosts. Conversely, could the host's microbiome drive the pathogenicity of gregarines in their hosts?

## Concluding Remarks

In summary, there is a clear association between gregarines and their hosts, leaning towards a codependent relationship. Despite being quite neglected when it comes to elucidating the evolution of parasitism in the Apicomplexa, gregarines form one of the most diverse groups of eukaryotes and have an important position within the apicomplexan tree that will provide further understanding of the evolution of parasitism within this clade. In our opinion, there is an urgent need to utilize new methods to explore the genetic and cellular composition of gregarines to identify properties that would allow the understanding of their relationship with, and selection of, their hosts. The first priority will be to produce both genomic and transcriptomic data of diverse members of the three orders of gregarines encompassing the whole spectrum of symbiosis (highlighted in Table S1, e.g., *Hoplorhynchus* sp. in Boreal bluet with positive effects on host and in Twelve-spotted skimmer with negative effects on host) and compare them with already existing data from other apicomplexan parasites (especially *Cryptosporidium* species [59]) and free-living apicomplexan relatives such as *Chromera* and *Vitrella* spp. [43]. These studies would identify the presence (if any) of factors related to the symbiotic adaptations of gregarines (see Outstanding Questions). Follow-up studies using host gut metagenomics and metatranscriptomics complemented with proteomics and metabolomics [3,60] to explore both the host specificity and the microbiome of invertebrates harbouring these gregarine species [61,62] would further elucidate the relationship and the roles of gregarines within their hosts (see Outstanding Questions). Due to their unique host specificity, the major goal would be the development of *in vitro* culturing systems based on invertebrate tissues. One of the go-to candidate hosts would be the mealworm, since *Gregarina* spp. have mutualistic, commensalistic, and parasitic effects on this host (Table S1). A long-term goal would be the development of an *in vitro* and axenic (animal- and tissue-free) culturing system [63,64] that would further permit the exploration of the basic cell and developmental biology of gregarines. Despite their importance, research on gregarines is progressing very slowly, and it will require researchers from various disciplines to come together and provide their expertise in expanding our knowledge on the evolution of parasitism in these microbes.

### Outstanding Questions

Why are gregarines the dominant endosymbiotic species in invertebrate hosts? What is their relationship with their host, and how can they influence the invertebrate biodiversity?

What triggers the host specificity in gregarine species? How do gregarines detect that they are in the right host to start the excystation process? Is the release of sporozoites a random process, or is there any form of control of the host's fitness involved? Could randomness explain the pathogenic effects of certain gregarine species in specific hosts, while being uninfluential in others?

Is there interplay between gregarines and intestinal microbiota within the host? Do gregarines influence its composition and diversity, or vice versa, and how does this occur?

Are gregarines the missing piece of the puzzle in our understanding of the evolution of parasitism in apicomplexans? If parasitism of vertebrates in apicomplexans did not evolve twice, is it possible that gregarines have been overlooked in vertebrate parasite surveys?

Is *Cryptosporidium* an actual gregarine, or are the current morphological and genomic evidence misguiding us? Would its phylogenetic position and relationships reshuffle when establishing rigorous and comprehensive phylogenomics analyses comprising various gregarine sequences and *Cryptosporidium* spp. data?

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## Supplemental Information

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