# Chapter 6

# Investigating the Diversity of Parasitic Protozoa using Gregarine Parasites of Invertebrates

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## Introduction

Gregarines are protozoan parasites in the phylum Apicomplexa. All members of this phylum (previously called Sporozoa) are parasites. The name Apicomplexa is derived from an ultrastructural feature called the apical complex (shown in Figure 1). This group includes many protozoan parasites of medical and veterinary importance including malaria, *Toxoplasma*, and *Cryptosporidium*. They have complicated life cycles that include different tissues in invertebrate and vertebrate hosts (see Fig. 2.) Because of this, and the fact that many of them are dangerous parasites of humans, it is difficult to study them using living material in the laboratory classroom.

Gregarines are Apicomplexan parasites that complete their life cycle within a single invertebrate host. Highly infected hosts, such as earthworms and mealworms, are easily obtained at bait shops and pet stores (as pet food) for use in the classroom. They are safe to use and the various life cycle stages are large so it is easier for students to observe. The gregarine life cycle includes a large vegetative stage often with interesting motility that is visible under the dissecting microscope. The large white gametocysts, often expelled from the host with feces, are easily visible under the dissecting microscope. If the cysts are allowed to develop, sporocysts dehisce. Even with the few commonly used host species, diversity in the protozoan parasite is evident. The gregarine life cycle can be related to that of the more medically important parasites such as malaria.

In addition to being easier to use in a classroom setting, gregarines have special features that make them interesting to study in their own right. I became interested in gregarines from my interest in cell motility and flagellar structure. The male gametes of many gregarines are flagellated. The gregarine trophozoites, the stage typically in the host intestine, have an amazing variety of active movements including amoeboid, euglenoid, pendular, and gliding. This exercise can be adapted for use in freshman biological diversity laboratories, to more advanced invertebrate biology courses, as well as parasitology and cell biology laboratories.

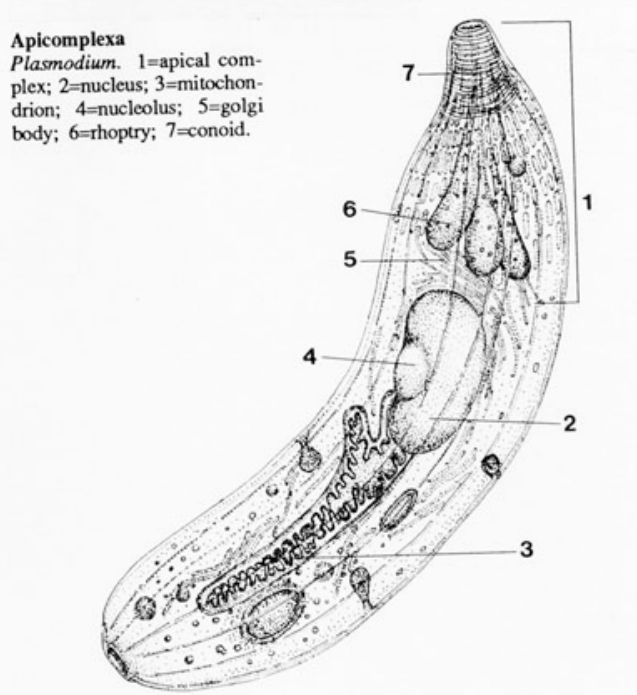


**Figure 1.** A diagram of *Plasmodium*, the causative agent of malaria. The apical complex is characteristic of the organisms in the phylum Apicomplexa.

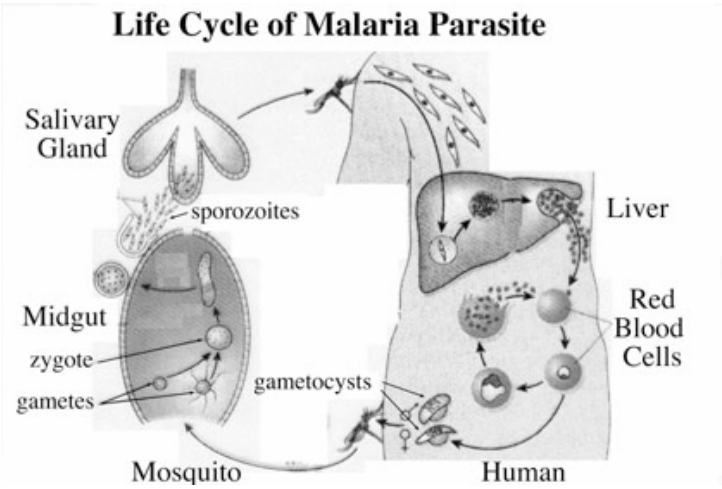


**Figure 2**. Life cycle of the malaria parasite includes different tissues in both vertebrate and invertebrate hosts.

Gregarines are species-specific parasites of a wide range of invertebrates from phyla Arthropoda (including crustaceans, insects, myriapods), Annelida (including polychaetes, oligochaetes and leeches), Nemertea, Mollusca, Echinodermata, and Urochordata. Gregarines are not often studied because they do not parasitize vertebrates, and do not even seem to cause serious damage to their invertebrate hosts. There are approximately 1,600 named species, but most have not been studied extensively. We can imagine that there are many more species yet to be described. This exercise will investigate the gregarine parasite *Monocystis* from the seminal vesicle of earthworms (Fig. 3) and intestinal gregarine parasites of tenebrid beetles (Fig. 4).

The life cycle of gregarines are illustrated in Figures 3 and 4. Infection begins with a host consuming a sporozoite, or sporocyst that releases sporozoites in the digestive tract. In the case of *Monocystis* in the earthworm, the sporozoite is motile and penetrates the intestinal wall and enters the testes. It then transforms into a growth stage called a trophozoite. In the case of *Monocystis* they are covered with host sperm tails and gain a ciliated appearance. As in many intestinal gregarines, the ones in the mealworm attaches to the host epithelium by a structure called epimerite. At this stage, the cell can grow quite large and be visible under the dissecting microscope. The sexual stage begins when two trophozoites (also called gamonts at this stage) of opposite sex associate in a process called syzygy. A walled cyst is produced around them to become a gametocyst. This cyst may be retained in the host as in *Monocystis* where we can see various developmental stages of the cysts. In the case of intestinal

gregarines, they are often expelled with the feces, as in the mealworm, marine polychaetes, and odonates (dragonflies and damselflies). The gametocysts are white spheres and can be quite large, so it is visible under the dissecting microscope and sometimes to the naked eye. We call them "pearls among poop!" Within this cyst, the two gamonts divide to form many gametes. The gametes from the two gamonts fertilize within the cyst to form diploid zygotes, called oocysts or sporocysts, which undergo meiosis to form haploid sporozoites, the infective stage. Thus for most of the life cycle, gregarines are haploid. Only when the two gametes fuse to form a zygote, do we have a diploid stage. Meiosis occurs soon to again form haploid cells.

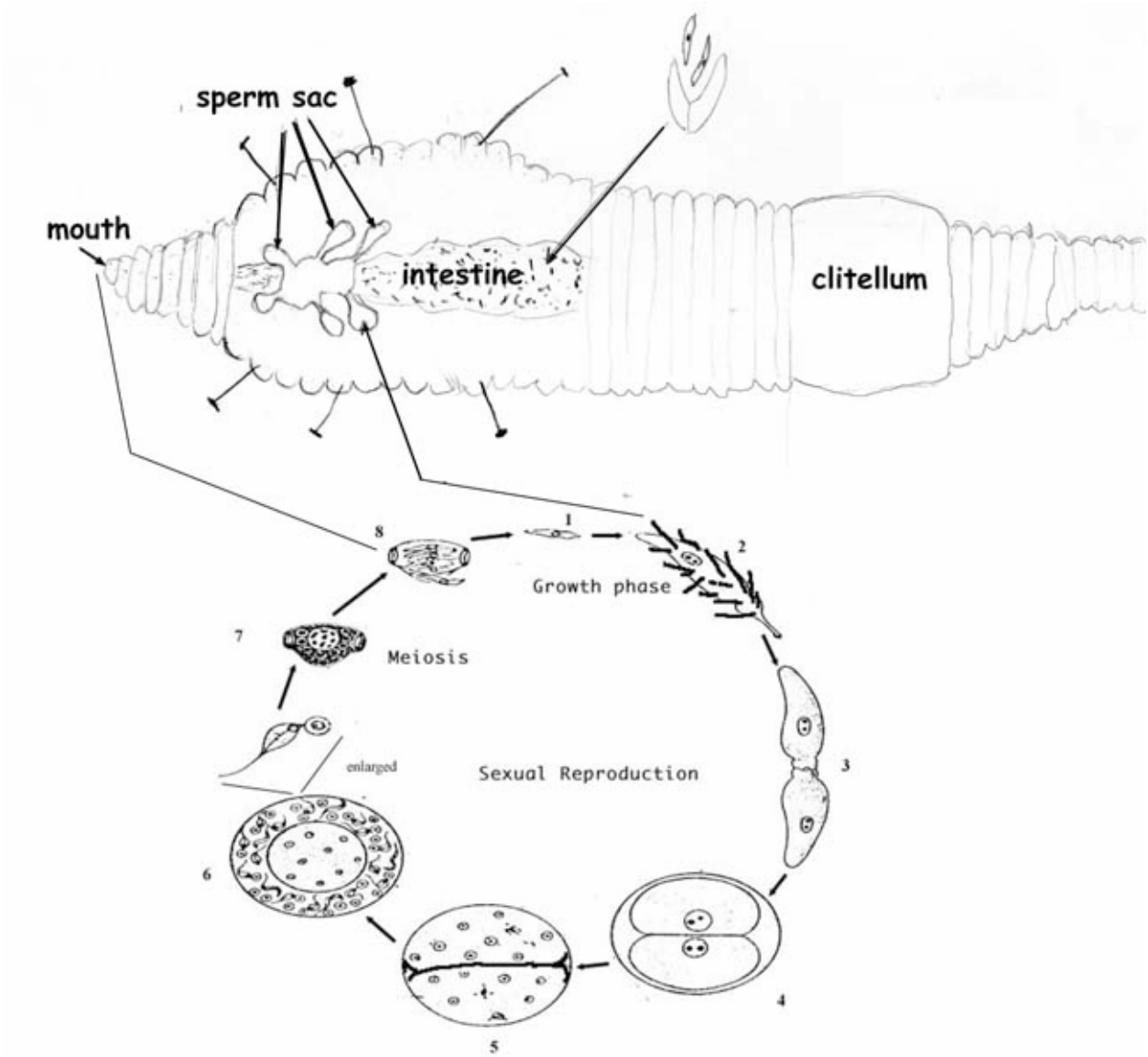


**Figure 3**. A diagram of the anterior part of an earthworm with the life cycle of *Monocystis* gregarines. The region from ~9th to the 20th segment has been pinned open. Only the intestinal tract and three pairs of sperm sacs are shown for clarity. The 3 cysts are fractions of mm in size while the trophozoites (growth phase) and sporocysts (meiosis) are much smaller.

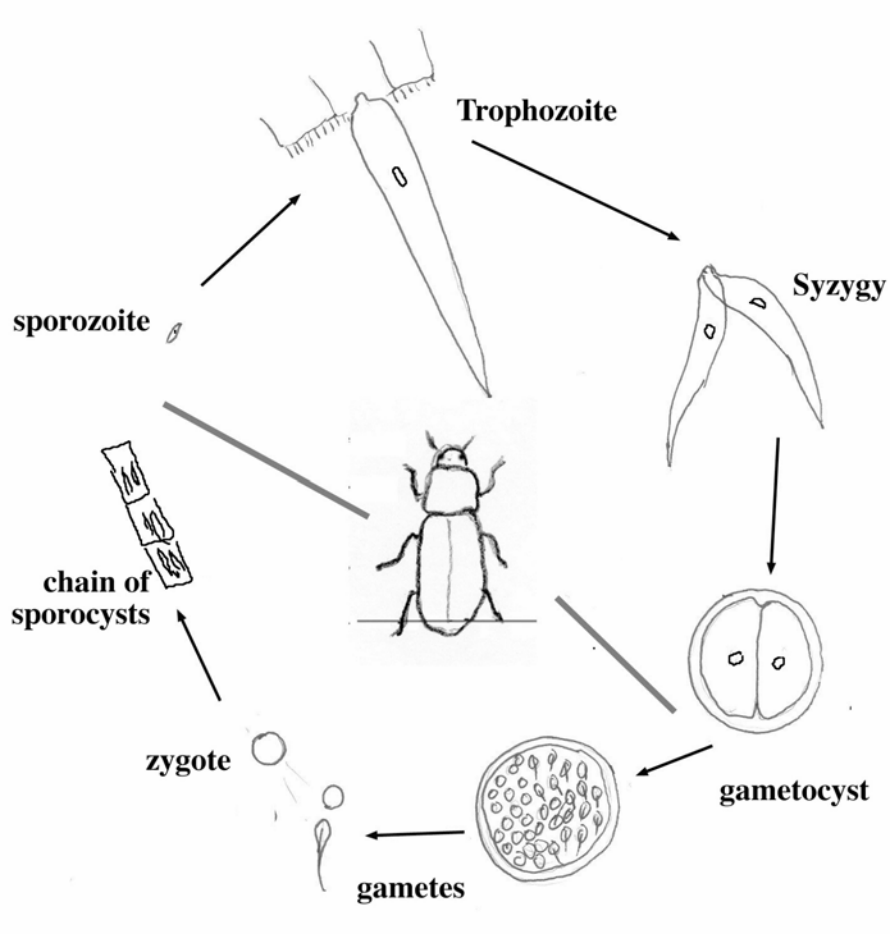


**Figure 4**. The life cycle of a mealworm gregarine. The cells around the beetle are roughly to scale, but not to scale with the beetle. The thin line at the posterior of the beetle indicates the region to be cut off with a pair of sharp scissors. The cells above the thick line out from the beetle are those inside the beetle whereas the ones below indicate those outside the host.

## Materials

**Supplies:**

- dissecting tray - 1 per group
- regular and fine forceps, - 2 each per group
- fine scissors - 1 per group
- dissecting needles - 2 per group
- scalpels, - 1 per group
- pins - 10-12 per group
- pasteur pipettes and bulbs. - 2 per group
- microscope slides and coverslips. - several per group
- regular and small plastic petri dishes. - few per group
- ruler - 1 per group

**Live organisms:**

- earthworm/nightcrawlers (from bait shops)
- mealworms (larvae and adult)

**Equipment:**

- dissecting microscopes - 1 per group
- compound microscopes with ocular micrometer if available - 1 per group
- microscope video camera (flex cam) and video monitor - one
- refrigerator or ice bucket (for anesthesizing beetles)

**Chemical supplies**

- Giemsa stain or methylene blue (1% aqueous)
- absolute methanol for fixation
- 7% ethanol for anesthetizing earthworms
- saline (0.7-0.9% NaCl)

# Student Outline

**Prelab assignment** (maximum 2 pages)

- Look up the life cycle of the malaria parasite, *Plasmodium*. On a diagram of *Plasmodium* life cycle, indicate the stages of the gregarine life cycle that might correspond to that of the malaria.
- The hosts we will be using may be infected by more than one species of gregarines. How would you go about trying to decide whether more than one species is infecting a single host?
- For a parasite, the host is an ecological landscape. What factors might determine the "ecological niche" of a gregarine species?

**Objectives**

- To make careful observations of gregarines
  - dissect an invertebrate host provided and find gregarine parasites.
  - document the different stages of parasites found

**Introduction and background information**

Members of the phylum Apicomplexa are all parasites. The name Apicomplexa comes from a complex cytoskeletal structure called the apical complex, which can be seen in electron micrographs (Fig. 1). This phylum includes parasites that cause major human and animal diseases such as malaria, coccidiosis, toxoplasmosis, *etc.* They have complicated life cycles that include different cells and organs in invertebrate and vertebrate hosts (Fig. 2). Because of this, and the fact that many of them are dangerous parasites of humans, it is difficult to study living material in the classroom laboratory. However, we can study a group of closely related parasites called gregarines that complete their life cycle within a single invertebrate host. Gregarines are species-specific parasites of a wide range of invertebrates from phyla Arthropoda (including crustaceans, insects, myriapods), Annelida (including polychaetes, oligochaetes and leeches), Nemertea, Mollusca, Echinodermata, and Urochordata. You can imagine that there are many species still undescribed. Today, we will have an opportunity to study the gregarine parasite *Monocystis* from the seminal vesicle of earthworms and intestinal gregarine parasites of tenebrid beetles.

**Procedures**

Procedures for the two different hosts are listed below. Concentrate on finding and describing gregarine parasites from one of the hosts. Pick either to do first (we will try to make sure at least one group does each host). Note the location in the host where gregarines are found and their size and shape at those locations. Depending upon how much time is remaining after studying one host thoroughly, you can either begin another, or share information with a group that chose a different host. You may find that another group has made interesting measurements or observations that you didn't for your gregarines, and you can go back and make those observations.

*Earthworms*

1. Anesthetize the earthworms by putting them in 7% ethanol solution for 5-10 minutes.
2. Place anesthetized earthworms in a dissecting tray.
3. Using sharp dissecting scissors or a scalpel, cut open the dorsal wall from the anterior to the clitellum. The ventral side has rows of setae that can be felt as slight rough edges, and the anterior is the rounded end. The clitellum is a wide band found near the 30th segment. Use dissecting pins to pin back the body wall and expose the internal organs.

4. The seminal receptacles are found around the 9th-10th segment while the seminal vesicles are three pairs of whitish sacs found posterior to them.
5. Place a couple of drops of saline on the area to prevent them from drying out.
6. Clip out a seminal vesicle and seminal receptacle and place them separately on a microscope slide. Try to tease apart the tissue with dissecting needles. In order to be able to see different stages, the tissue must be thin enough to see through. If necessary, add drops of saline to dilute out the host tissue.
7. Place a coverslip on the tissue and gently press with a rolling motion of a dissecting needle or pencil. Try not to slide the coverslip during this process.
8. Small refractile lemon-shaped structures are sporocysts, and if developed, contain sporozoites. Trophozoites are larger cells covered with host sperm tails that make them appear hairy. Larger cysts can contain, in order of development stage, two large gamonts, many gametocysts, or many lemon-shaped sporocysts.

*Mealworm Beetles*

1. Anesthetize beetles by putting them in the refrigerator 5-10 minutes. This will slow them down but not kill them or the parasites they contain.
2. Take a beetle and clip off the posterior tip with scissors and place on an inverted plastic petri dish.
3. Using two forceps, grasp the head and thorax and gently pull apart. The intestine should come out attached to the head.
4. Cut off the intestine and place on a microscope slide with a drop of saline.
5. Tease apart the intestine using fine dissecting needles. Trophozoites and gamonts in syzygy may be visible under the dissecting microscope. Note any motion of the tropohozoites and the manner of syzygy.
6. For observation at higher magnification, place a coverslip and gently press with a rolling motion of a dissecting needle or pencil. Try not to slide the coverslip during this process.

Gametocysts of many intestinal gregarines are excreted with the feces and appear as white spheres. We have some feces in petri dishes from beetles that had been in them overnight. Can you find the gametocysts? Because gametocyst development takes many hours to days, we also have gametocysts that had been collected previously. Squash the gametocyst in saline and observe under the compound microscope. Compare the older gametocysts with younger gametocysts. We also have some gametocysts that have dehisced. Compare the previous gametocysts to those that have dehisced under the compound microscope.

## Notes for Instructors

This laboratory study is suitable for a number of different laboratory courses from general surveys of biological diversity, protozoology, to parasitology. Because the trophozoite stage of gregarines in the host is often quite large they can be useful for a general cell biology laboratory. Their interesting motility can also be used in investigative exercise in advanced cell biology.

**Choice of hosts species**

Gregarine parasites are found in almost all invertebrate groups. The organisms for this laboratory exercise were chosen because they are readily available and are fairly dependably infected with gregarines. Earthworm seminal vesicles are highly infested throughout the year. Mealworms (as larva) are available from pet shops for pet food. Both larvae and adults are typically infested, though pupae are not.

If other invertebrates are readily available, they may be worth searching for gregarines. For example, a number of marine polychaetes have a large number of gregarines in their intestinal tract. If cockroaches are available, they too have well characterized gregarines. One can test for infestation of many hosts without dissection by placing 10-20 of them in a large petri dish overnight in the dark. If they are infested and shedding gametocysts, they are readily observed as white perfectly spherical objects among the feces.

**Microscopic observation**

It is recommended that material first be observed live and unfixed. These gregarines are surprisingly large for single-celled organisms, and many stages are recognizable under the dissecting microscope. The feeding or trophozoite stage continues to move even after separation from the host tissue in an aqueous medium that is not optimized for long-term survival. Though we mention Giemsa and Methylene Blue, almost any histological stain will work for gregarines.

**Making an inexpensive set of miniature dissecting needles**

Dissecting small insects or other invertebrates often requires finer tools than one typically has for animal dissections. The following are directions for making fine dissecting needles using cheaply obtained items. This procedure involves sharp objects and can be hazardous. The instructor may wish to make these for the students.

*Supplies*

- 1/4" dowels cut to ~3" length with a hole (or handles from old dissecting needles)
- 000 insect pins
- 5-minute Epoxy
- disposable plastic 15 mL conical centrifuge tubes with caps

*Procedure to make miniature dissecting needles*

- Dip the knob end of a 000 insect pin into 5 minute epoxy and place into the hole in the end of the dowel. Let harden 15 minutes before use.

Once the epoxy has hardened, these miniature tools can be stored and safely carried in disposable plastic 15 mL conical centrifuge tubes with caps.

## Acknowledgements

We thank Dr. John Janovy, Univ. of Nebraska, Lincoln, for introducing us to mealworm gregarines and gregarines in general. We also thank Dr. Armand Kuris, Univ. of Calif. Santa Barbara for letting us use material from his parasitology laboratory course manual.

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