Part II

PROTOZOA, MYXOZOA, MESOZOA

PROTOZOA

9

Protozoa

Apicomplexa

The Coccidia Proper:

Important Apicomplexa Other than Haemoprotozoa

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Phylum Myzozoa

Subphylum Apicomplexa

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Chapter 9

The Coccidia Proper: Important Apicomplexa Other than Haemoprotozoa

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History of the Term Apicomplexa

Taxonomy addresses the principles of scientific classification by discovering, observing, defining characters, ordering into groups, naming individual organisms that are clearly different within those groups, and archiving type specimens as appropriate in accredited museums. Historically, all living things defined as animals (that is, non-plants), were placed in 1 of 2 groups: Protozoa (meaning single-celled protists) or metazoa (meaning multicellular animals). Omitting hierarchical names (kingdom, phylum, class, and so on) for the moment, all protozoa were ordered into 1 of 4 groups based on how they moved, or didn't: Ciliates (which have cilia), amoeba (which have pseudopodia), flagellates (which have flagella), and a catch-all category called the Sporozoa, most of which (but not all) had spores and some of which (myxosporidia and microsporidia) were not even remotely related to the spore-formers.

As knowledge increased, the name Sporozoa became unwieldy because it did not suggest or represent true evolutionary relationships between the organisms included therein. The widespread use of Transmission Electron Microscopy (TEM) for biological specimens began in the 1950s and continued throughout the 1960s and 1970s; many of these studies examined the fine structure of zoites belonging to a plethora of different protozoans. Eventually, a pattern began to emerge that revealed several common, consistently-shared structures (for example, polar rings, rhoptries, micronemes, and often a conoid) at the more pointed end (now termed anterior) of certain life stages (Figure 1). When present, these structures, in whatever combination, were termed the apical complex. At that time, protozoologists working on parasites sought a more phylogenetically relevant suite of characters to define their organisms, and Norman D. Levine, from the University of Illinois, came up with the name Apicomplexa to unify them. This complex structure is now known to be the focus of events during host cell penetration and establishment of the parasite within the cells of the host.

Introduction to the Apicomplexa

The protozoan group Apicomplexa Adl et al., 2005 (Levine, 1980) contains many obligate intracellular parasites including such diverse organisms as coccidia, gregarines, haemosporoids, piroplasms, and cryptosporids, all united not by their biology or life histories, but by the presence of their unique apical complex. This complex collection of protozoans is subdivided into 2 major assemblages based on the presence or absence of a conoid in their apical complex. The Aconoidasida Mehlhorn et al. 1980 [= Hematozoa Vivier, 1982] all lack a conoid in their asexual motile stages, and include the Haemosporida Danilewsky, 1885 (for example, *Plasmodium, Haemoproteus, Leucocytozoon,* and others) and Piroplasmorida Wenyon, 1926 (for example, *Babesia, Theileria*, and others).

Members of the second major grouping, Conoidasida Levine, 1988, all have a complete apical complex that includes a hollow, truncated conoid in all or most of their asexual motile stages, along with other unifying features. This paraphyletic lineage includes 3 groups: Gregarinasina Dufour, 1828; the monogeneric family Cryptosporididae Tyzzer, 1907; and Coccidia Leuckart, 1879 according to Adl et al. (2012). Of the 2 Conoidasida groups that will not be covered in detail here, the gregarines parasitize invertebrates, and *Cryptosporidium* species, which were once considered to be atypical Coccidia, are most closely related to the gregarines and not the Coccidia (Cavalier-Smith, 2014; Thompson et al., 2016).

Before delving into the Coccidia, the history of taxonomic placement of the former Cryptosporididae will be discussed briefly. Bull et al. (1998) first noticed there was serological cross-reactivity between anti-*Cryptosporidium* monoclonal antibody and sporocysts of the gregarine *Monocystis*, an observation mostly overlooked—or ignored—at the time. The next year, when sequencing SSU rDNA, Carreno et al. (1999) inferred that *Cryptosporidium* was more closely related to gregarines than to Coccidia by phylogenetic analysis of apicomplexan parasites. Based on this and

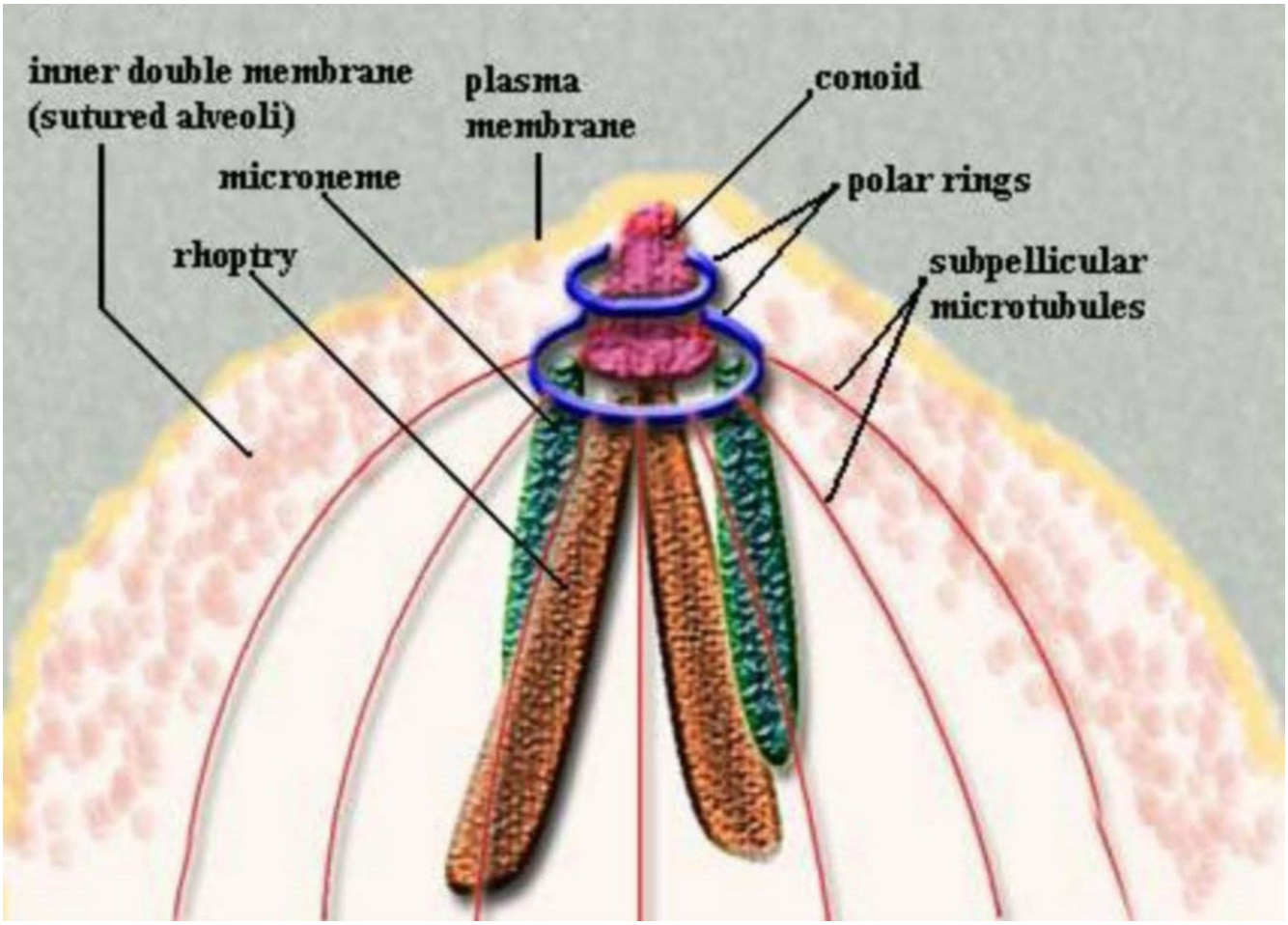


Figure 1. Apical complex structures at the anterior end of a coccidian zoite. Image source: Clowes et al., 2006. License: CC BY.

other molecular congruences, and on biological and behavioral similarities, Cavalier-Smith (2014) established a new subclass, Orthogregarinia, for *Cryptosporidium* and its most closely related gregarines, which include epicellular parasites of vertebrates possessing a gregarine-like feeder organelle and lacking an apicoplast (which is a relict, non-photosynthetic plastid found in most apicomplexan parasites). In addition to the SSU-rDNA sequencing evidence, *Cryptosporidium* shares biological features with gregarines including its epicellular location, connection to the host cell via a myzocytosis-like feeding mechanism, heterogeneity of trophozoite cell shape, and other structural similarities (see Thompson et al., 2016). Gliding movements seen in different trophic stages of *Cryptosporidium* species are behavioral features that also are similar to gliding movements exhibited by some gregarines (Borowski et al., 2008; 2010; Valigurová et al., 2013).

The Coccidia

Coccidia are united by having mature gametes that develop intracellularly, microgametocytes that usually produce many microgametes, and non-motile zygotes that mostly contain sporocysts within their oocysts. There are 2 Coccidia lineages: **Adeleorina Léger, 1911** and **Eimeriorina Léger, 1911**. The Adeleorina has about 7 families, 2 of which each contain a genus of important parasites of vertebrates, Hepatozoidae Wenyon, 1926 (genus *Hepatozoon*) and Klossiellidae Smith and Johnson, 1902 (genus *Klossiella*). The Eimeriorina has 10–12 recognized families, 2 with multiple genera containing important parasites of vertebrates. The Eimeriidae has about 20 genera, but only 6 will be mentioned, to illustrate their diversity, namely, *Acroeimeria*, *Caryospora*, *Choleoeimeria*, *Cyclospora*, *Eimeria*, and *Isospora*. The Sarcocystidae has 7 genera of which 5 have extremely important parasites of humans and/or their domestic animals, namely, *Besnoitia*, *Cystoisospora*, *Neospora*, *Sarcocystis*, and *Toxoplasma*.

Important Genera, Relation to Other Species, and Basic Life Histories

The apicomplexan genera with species that are important parasites of humans and/or their domestic, companion, and wild animals are highlighted in this section in the same taxonomic sequence outlined above:

Conoidasida Levine, 1988
Coccidia Leuckart, 1879
Adeleorina Léger, 1911

The Adeleorina is a poorly understood group of apicomplexan parasites. Members are united biologically by use of syzygy, a characteristic method of gamete formation by which both macro- and microgamonts are pressed together during their development (Adl et al., 2012). The Adeleorina has 7 families of coccidia and includes those with both homoxenous and heteroxenous life cycles (Barta, 2000). In heteroxenous species, the conjugation of gamonts and subsequent sporogony most often occur within an invertebrate definitive host and (mechanical) vector; the oocysts formed contain numerous sporocysts, and sporozoites are found in the hemocoel of the definitive host (Craig, 2001). Once the vector is ingested, sporozoites are released, after which they penetrate the gut of the vertebrate intermediate host and enter the bloodstream to reach leukocytes and cells throughout the body where they undergo merogony. Many of the species in this group have morphologically distinct meronts and merozoites during their asexual reproduction, which occurs in the vertebrate (that is, intermediate) host. The first-generation meronts (M_1) produce large merozoites (m_1) that are thought to initiate a second round of merogony in which the M_2 produce smaller m_2s, which then become the progenitors of gamonts (Barta, 2000). Merogony in the tissues ultimately gives rise to gamonts in white blood cells (WBC) and tissue cysts; these tissue cysts may be a stage that can be transmitted by predation, but this remains to be determined (Craig, 1990; 2001).

Family Hepatozoidae Wenyon, 1926

This family has a single genus, *Hepatozoon* Wenyon, 1926b, with more than 300 described species (Baneth et al., 2007; Ivanov and Tsachev, 2008). Species in this genus infect various vertebrates including amphibians, reptiles, birds, and mammals, which are their intermediate hosts. The definitive hosts for these species are invertebrates that include mites, ticks, and various insects, and infection of the vertebrate host occurs when it ingests the infected invertebrate (not by its bite). Barta (2000) suggested the genus is paraphyletic. One important species in this genus, which can parasitize a favorite companion animal, the domestic dog, will illustrate the biology of these species.

Genus *Hepatozoon* Wenyon, 1926 (Figure 2).

Hepatozoon canis (James, 1905) Wenyon, 1926, can cause serious, life-threatening illness in vertebrates. In addition to dogs, it has been found parasitizing cheetahs, coyotes, jackals, foxes, hyenas, lions, and leopards (each as intermediate hosts) and has a worldwide distribution wherever its definitive host, the brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806), is found. Note that other tick species also can serve as definitive hosts. Its prevalence in infected canid populations often is modest but also may be quite high. For example, Conceição-Silva et al. (1988) found 143 of 301 (48%) red foxes in Portugal to be infected while only 50 of 1,752 (3%) domestic dogs from the same area were infected. O'Dwyer et al. (2001) examined blood smears of dogs from rural areas of 7 counties in Rio de Janeiro state, Brazil, and identified *H. canis* in 98 of 250 (12%) dogs. Cardoso et al. (2014) detected *H. canis* in 68 of 90 (76%) red foxes from 8 districts in Portugal, using both molecular (PCR amplification of 18S rRNA gene fragments) and histopathological sections of multiple tissues (bone marrow, heart, hind leg muscle, jejunum, kidney, liver, lung, popliteal or axillary lymph nodes, spleen, and/or tongue). Furtado et al. (2017) collected blood samples from domestic dogs from 3 regions of Brazil; 81 of 129 (63%) dogs were positive for *H. canis*, as determined by PCR nucleotide sequences of the 18S rRNA gene of *Hepatozoon*.

In the life cycle of *Hepatozoon canis* in vertebrates (Figure 2), monozoic cysts have been found in the spleen, meronts and merozoites in the spleen, lymph nodes, lungs, liver, bone marrow, and gamonts and/or gametocytes in the cytoplasm of neutrophils and monocytes. Once ingested by the tick definitive host, gamonts need about 24 hours to free themselves from vertebrate WBCs and soon thereafter they align side-by-side in syzygy. At 48 hours in the tick, 2 types of cells are present: Elongated cells with an eccentric nucleus, presumed to be microgametes, and more rounded cells, also with an eccentric nucleus, presumed to be macrogametes. At 4 days, zygotes (early oocysts) are formed; by 5 days oocyst wall and sporocyst formation have begun (Baneth et al., 2007) and these stages are extracellular, not within tick host cells. Vincent-Johnson et al. (1997) measured *H. canis* oocysts and said they were mostly spheroidal, 215 × 193 (160–325 × 138–258) µm with sporocysts that were 36 × 26 (29–41 × 17–30) µm.

Infection with *Hepatozoon canis* in dogs (and other vertebrates) ranges from being asymptomatic with low-level parasitemia, to a severe, life-threatening illness with fever lethargy, anemia, and emaciation in animals with very high parasitemia (Baneth et al., 2007). Sakuma et al. (2011) listed the characteristic hematological abnormalities in *H. canis*

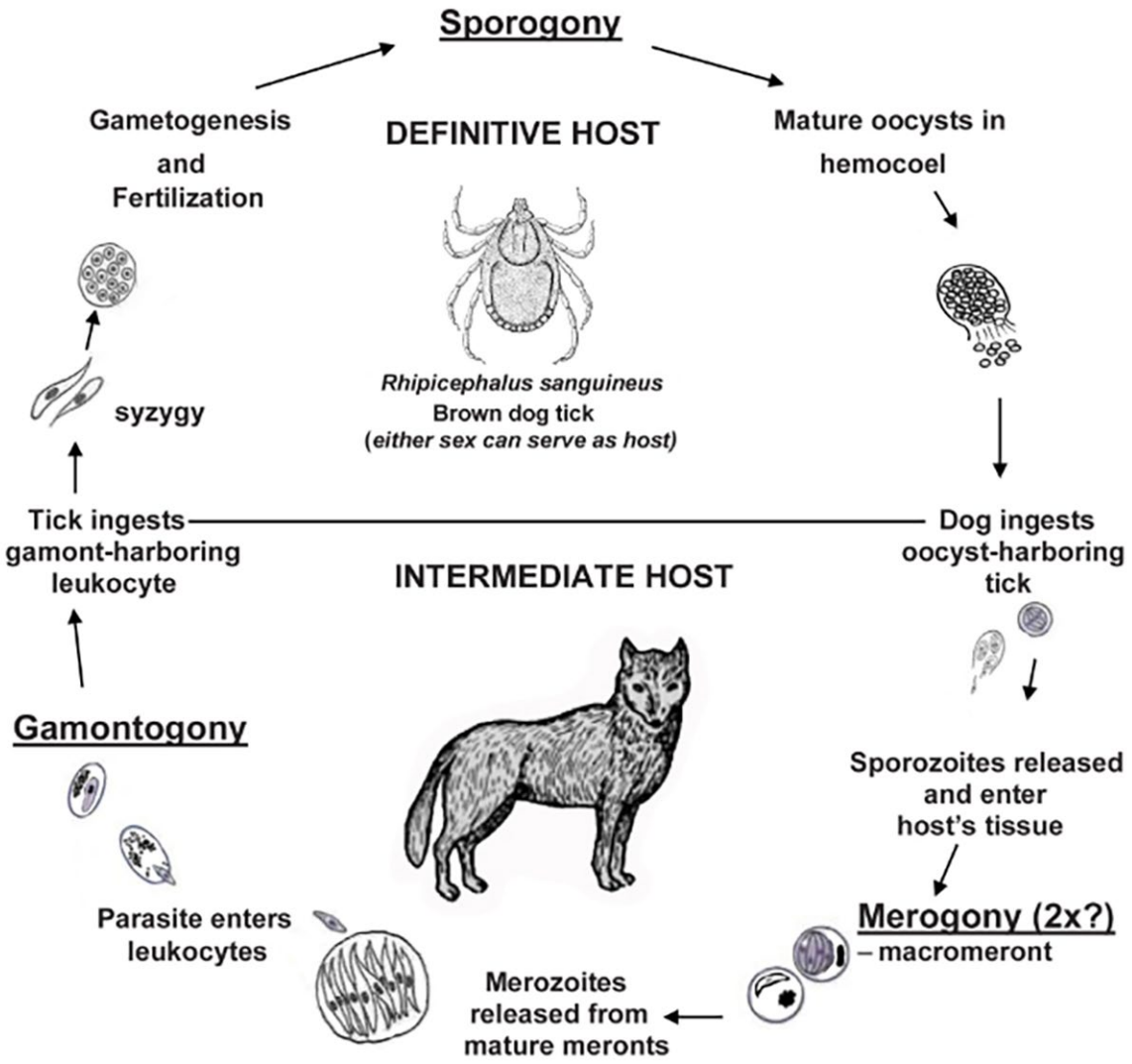


Figure 2. Diagrammatic drawing of the life cycle of *Hepatozoon canis* in dogs. Image sources: Tick, Pratt and Littig, 1962. Dog, V. Rausch, 1952. Other Figures, originals by S. L. Gardner, 2023. Tick image public domain; all other images, CC BY-NC-SA 4.0.

infections to include nonregenerative anemia, thrombocytopenia, neutrophilia, hyperproteinemia, hypoalbuminemia, polyclonal gammopathy, and increased concentrations of serum creatine kinase and alkaline phosphatase.

Family Klossiellidae Smith and Johnson, 1902

This family also has but 1 genus, *Klossiella* Smith and Johnson, 1902, and it contains about 18 named species that infect primarily mammals, in which it invariably undergoes asexual and sexual development in the kidneys. For example, *K. muris* is found in the kidneys of house and lab mice (*Mus musculus*), *K. cobayae* in the capillaries of the guinea pig (*Cavia porcellus*) kidney, and *K. equi* in the kidney of asses (*Equus asinus*) and horses (*Equus caballus*) (Levine, 1973; Levine and Ivens, 1965). Levine and Ivens (1965) reviewed the highlights of Smith and Johnson's (1902) discovery and

Box 1. *Hepatozoon* species — Learn More

Interested readers can find more detailed information on this and other *Hepatozoon* species in dogs, cats, and other carnivores in Duszynski et al. (2018). If interested in various tissue stages, a picture of a meront in the spleen of a dog from the Philippines is shown in Vincent-Johnson et al. (1997; Figure 5); developmental stages in the tick and scanning electron microscopic images of oocysts and sporocysts in ticks are found in Baneth et al. (2007; Figures 2–13 and Figures 14–17, respectively).

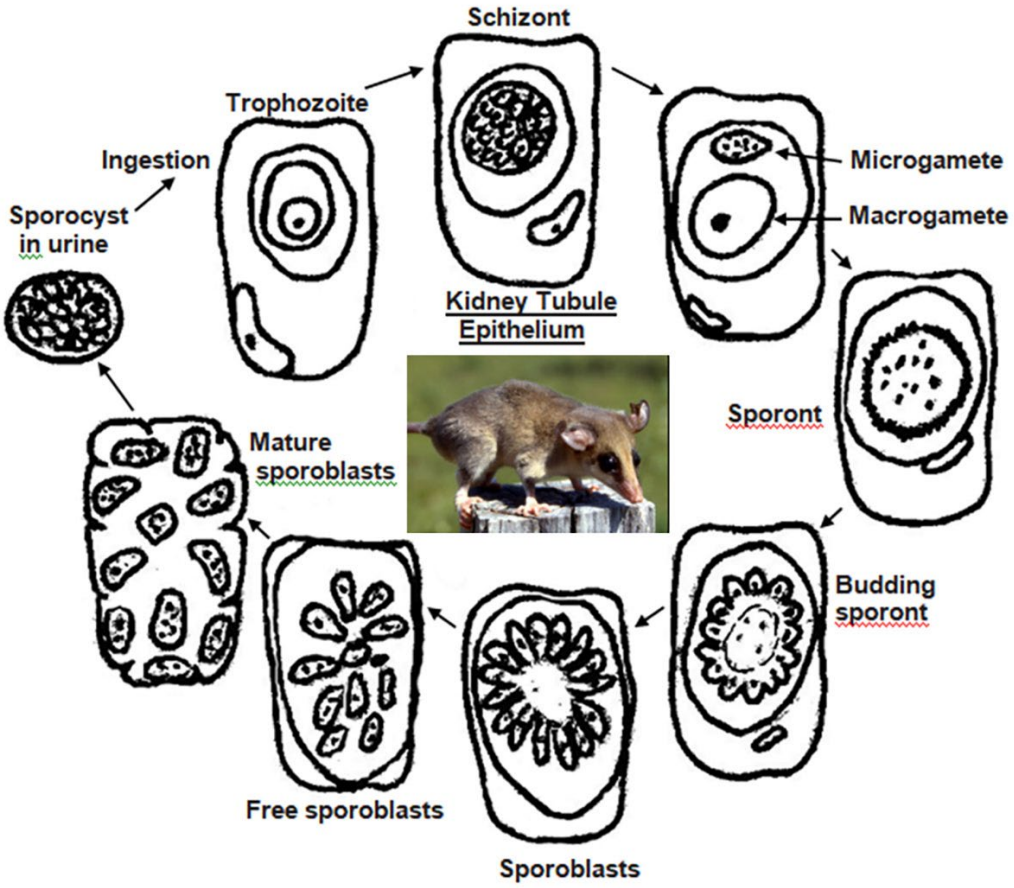


Figure 3. Diagrammatic drawing of the life cycle of *Klossiella tejerai* in opossums. Drawings by Duszynski. Photo by S. L. Gardner, 1993. License for all: CC BY-NC-SA 4.0.

the known developmental stages of this unusual coccidian. One example of a *Klossiella* species that infects the common opossum will suffice to illustrate this very interesting parasite family.

Genus *Klossiella* Smith and Johnson, 1902

Scorza et al. (1957) described *Klossiella tejerai* from a single common opossum, *Didelphis marsupialis* (Linneas, 1758), in Venezuela. To date, *K. tejerai* only has been found in 2 other instances: In 4 of 10 (40%) of *D. marsupialis* in

Box 2. A Cautionary Example — Learn More

Edgcomb et al. (1976) were among the first investigators to talk about pathological changes due to *Klossiella* species. They said, "Passage of schizonts (= meronts) and merozoites through the glomerular membranes occurs without inflammation and hemorrhage. These forms of the parasites evidently have membranes that permit their passage through the entire glomerular wall with restoration of the wall to an intact functional state after passage" (p. 316–317). This seems an odd interpretation from observing just a few tissue sections. It can be envisioned how merozoites can penetrate cell membranes, but not meronts. They went on to say, "The invasion of tubular epithelial cells by gametes, particularly by macrogametes, is associated with ballooning necrosis of the invaded cells" (p. 317). Spitz dos Santos et al. (2014) cautioned that Edgcomb et al. (1976) may have misinterpreted their photomicrographs.

Panama (Edgcomb et al., 1976) and in 1 of 20 (5%) in big-eared opossums, *Didelphis aurita*, from Brazil (Spitz dos Santos, 2014). It is surmised that both asexual (merogony) and sexual (gamogony) stages are found within epithelial cells of the kidneys and associated ducts and tubules. The life cycle is direct (Figure 3) with very large oocysts, 72 × 47 (57–103 × 36–57) µm, that are irregular in shape, sporulation (sporogony) is endogenous producing 12–30 sporocysts, 20.4 × 12.7 (19–22 × 12–14) µm, each with 8–20 sporozoites.

Box 3. *Klossiella* Species — Study It

Clearly, studying *Klossiella* species in the kidneys of vertebrates is an area ripe with potential rewards for new information. Parasitologists should begin to incorporate collecting urine into their field protocols to gain a sense of what oocysts and sporocysts of *Klossiella* really look like, and what variation can exist among species. Collecting kidney and related tissue samples for squash preparations/smears to be stained, and blocks of kidney to be fixed, embedded, sectioned, and prepared for histological examination (light microscopy (LM), transmission electron microscopy (TEM), or scanning electron microscopy (SEM)) will be critical. It will be an innovative milestone when someone finally infects several specimens of a vertebrate species with *Klossiella* oocysts/sporocysts, and then traces the sequential development over time of a complete life cycle within their kidneys. And, of course, it is imperative that DNA be collected and sequenced to gain an exact sense of the nature and affinity of these very interesting parasites—about which so little is known—to other species groups of the Apicomplexa. There are certainly a vast number of potential and obvious research projects available within this system to explore and problems to solve. This presents a wonderful opportunity, especially for graduate students who are teaching, to recruit undergraduates to help them with both field and lab work.

Eimeriorina Léger, 1911

Eimeriidae Minchin, 1903

The Eimeriorina contains species that all undergo merogony (asexual), gamogony (sexual), and sporogony (spore formation) during their life cycle. Members of the Eimeriidae all are homoxenous (direct life cycle), with merogony, gamogony, and the formation of oocysts occurring within the same host. Oocysts then leave the host, via the feces, and usually are unsporulated (= undeveloped, non-infective), but with a few exceptions (*Choleoeimeria*). The development of a genetically determined number of sporocysts and sporozoites within each oocyst occurs outside the host if/when environmental conditions (oxygen, moisture, and temperature) are appropriate (see Figure 4).

Genera *Acroeimeria* Paperna and Landsberg, 1989 and *Choleoeimeria* Paperna and Landsberg, 1989 (Figure 4)

The sporulated oocysts of *Acroeimeria* and *Choleoeimeria* are similar to those of *Eimeria* (see below) in that they all possess 4 sporocysts, each with 2 sporozoites, but all of their sporocysts lack a Stieda body, while most eimerians produce sporocysts with a Stieda body. Bovee and Telford (1965, page 93) were the first to see a possible relationship between the shape of lizard *Eimeria* spp. oocysts and their site of infection when they wrote,

> *Eimeria* spp. of lizards which form nearly spherical or elliptical oocysts with index 1.4 or less are inhabitants of the small intestine (if site of infection is known). Those of greater index, that is, long-ellipsoid or cylindrical form, are parasites of the

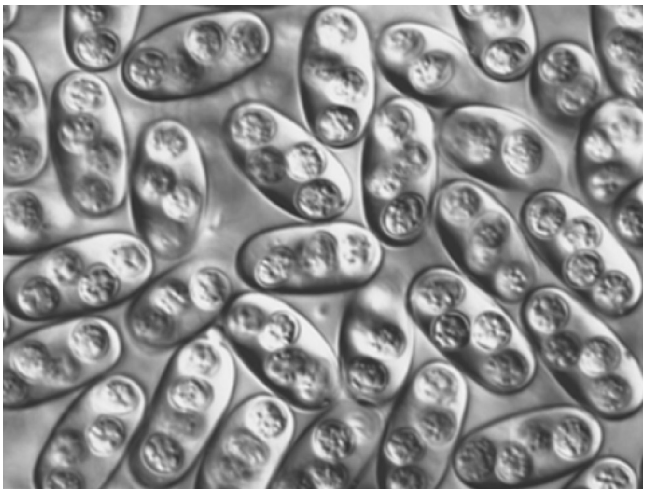
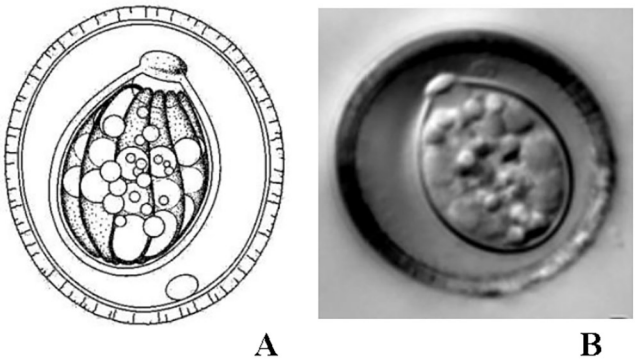


Figure 4. Elongate-ellipsoidal sporulated oocysts of a *Choleoeimeria* species in the bile duct and gallbladder from a colubrid snake, *Masticophis flagellum* from Texas. Original photomicrograph, Duszynski and Upton, 2010. License: CC BY-NC-SA 4.0.

biliary tract and particularly the gall bladder. The significance of this size-shape relationship to site is unknown.

Three decades later, Paperna and Landsberg (1989) reexamined the relationship between location of endogenous development of *Eimeria* spp. in geckos, their shape, and sporocyst structures. To accommodate their observations, they erected 2 new genera, *Acroeimeria* and *Choleoeimeria*. Both of their new genera had the general characteristic of the Eimeriidae (above). However, they defined *Acroeimeria* to have round or ovoidal oocysts with a length/width (L/W) ratio < 1.8, and all of their endogenous development (meronts, gamonts) "at the microvillous zone of the host cell and enclosed in the host cell microvillous boundary, causing the host cell to extend above the intestinal mucosal surface," and sporulation was exogenous. *Choleoeimeria* was defined to have cylindroid to ovoidal oocysts with a L/W ratio always > 1.4 (up to 2.2), endogenous development (meronts, gamonts) in the gallbladder (as far as was known then), development that induced hypertrophy and displacement of epithelial host cells above their original cellular layer, and sporulation was endogenous in the gallbladder and gut lumen. Thus, *Acroeimeria* sporulated oocysts looked like *Eimeria* oocysts, but their endogenous developmental processes were unique.

Localization of endogenous development in the microvillous zone of intestinal epithelial cells was described earlier in fish eimerians by Dyková and Lom (1981) who proposed a new genus, *Epieimeria*, to accommodate these presumably epicytoplasmic piscine eimerians. However, Benajiba et al. (1994) noticed that *Epieimeria* showed both epicytoplasmic and intracytoplasmic endogenous development, and Paperna (1991), using TEM showed both epicytoplasmic and intracytoplasmic endogenous stages that develop within a parasitophorous vacuole, which makes them only intracytoplasmic. Thus, epicytoplasmic endogenous development did not occur in fish *Epieimeria* and this urged Benajiba et al. (1994) to suppress the genus name and reassign all *Epieimeria* species back to the genus *Eimeria.* Several years later, Lainson and Paperna (1999), for unexplained reasons, changed their original definition of *Acroeimeria* slightly by stating that it, "Develops immediately beneath the brush-boarder of the intestinal epithelial cell" (page 151), and this begs the question, whether or not *Acroeimeria*, as defined to have only epicytoplasmic endogenous stages (Paperna and Landsberg, 1989), also should be suppressed. We now know that some species of *Acroeimeria* exhibit both epicytoplasmic (typical of *Acroeimeria*) and intracytoplasmic (typical of *Eimeria*) endogenous development (unpublished data), a situation very similar to the piscine *Epieimeria* story. No one yet has done a careful molecular characterization of *Acroeimeria* versus other coccidians in saurian species.



Figures 5A–B. A) Line drawing of the sporulated oocyst of *Caryospora duszynskii*. B) Photomicrograph of a sporulated oocyst of *C. duszynskii*. Both Figures from a colubrid snake, in Duszynski and Upton, 2010. License: CC BY-NC-SA 4.0.

The *Choleoeimeria* story is much simpler. Development of these species in the gallbladder and associated ducts, with the production of elongate-ellipsoidal or cylindroidal oocysts (Figure 4) that mostly sporulate endogenously seem to be conditions accepted by most of those who study lizard parasites, so far. Assigning species to *Acroeimeria* is more difficult and requires studying not only oocyst morphology, but structural information must be supported by information on the location and stages of endogenous development, and (multiple) gene sequencing whenever possible. We suggest as a practical matter, that unless information on endogenous development and/or partial gene sequence can be obtained to support morphology of saurian coccidia recovered from the feces, the species names used should almost automatically be placed in the *Eimeria*.

Genus *Caryospora* Léger, 1904 (Figures 5A–D)

The *Caryospora* genus is a really intriguing group of apicomplexan parasites. *Caryospora* species are mostly parasites of reptiles (predominantly snakes, but also lizards, turtles) and birds, and 1 species has been reported from mammals. Species assigned to this genus have 2 unusual features. First, their sporulated oocysts (Figures 5A–B) have only 1 sporocyst, which contains 8 sporozoites. Interestingly, those species described from reptiles almost always have a prominent Stieda body/substieda body complex while most of those caryosporans described from birds do not have a Stieda body/substieda body at the more pointed end. Although some species in this genus utilize life cycles similar to *Eimeria* and *Isospora* species, the second unique feature is that several *Caryospora* species from snakes are facultatively

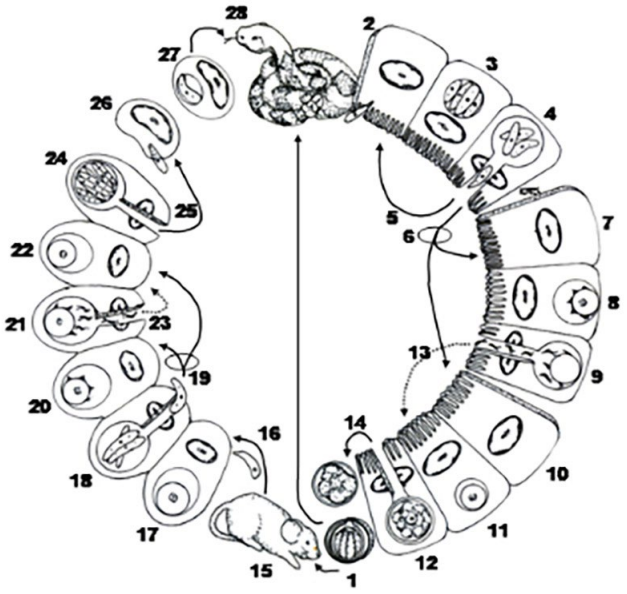


Figure 5C. Life cycle of a *Caryospora* species with both direct and facultatively heteroxenous life cycle components. 1) Typical sporulated oocyst which may be ingested by the snake definitive host in which case the parasite will undergo an enteric life cycle similar to *Eimeria* and *Isospora* species. 2) Sporozoites excyst in the intestine, penetrate epithelial cells, and then form meronts (3) with merozoites that rupture from the host cell (4). These may invade other epithelial cells to undergo several merogonous stages (5), or they may penetrate epithelial cells to produce micro- (6–9) or macrogametocytes (10–12), also typical of enteric coccidia. After fertilization (13) an unsporulated oocyst is formed which then ruptures from the host cell and is shed in the feces of the snake (14). Rodents are the typical secondary host for the facultatively heteroxenous part of the life cycle. When sporulated oocysts are ingested by a rodent (15), sporozoites excyst in the intestine, cross the gut wall, and become disseminated throughout the dermal tissues of the host, probably via the bloodstream. (16) In these cells, at least 2 asexual generations occur (17–19) followed by the sexual stages (20–22). Following fertilization, thin-walled oocysts are formed (24) and 8 sporozoites develop within a membrane, in the absence of a true sporocyst. These rupture from the host cell containing the oocyst, enter macrophages and/or fibroblasts, and are termed caryocysts (25–27). When eaten by the snake host (28), sporozoites are released from caryocysts and development continues in a manner thought to be identical to that known to occur when sporulated oocysts are ingested. Original Figure from Duszynski and Upton, 2010. License: CC BY-NC-SA 4.0.

heteroxenous. In this type of life cycle, both an enteric phase in a snake host and a non-intestinal phase in rodents have been described (Figure 5C).

After asexual and sexual multiplication in snake intestinal epithelial cells, typical of that known for other enteric coccidia, unsporulated oocysts are passed in the feces, but

Figure 5D. Photomicrograph of experimentally-infected (left) and control (right) laboratory mice showing the swelling of dermal tissue about 12 days post-infection with 250,000 sporulated oocysts of *Caryospora simplex*. Source: Duszynski and Upton, 2010. License: CC BY-NC-SA 4.0.

patency may last for months, or even a couple of years; this suggests that either some enteric recycling of asexual stages is occurring or that oocysts are retained deep within host tissues for an unusually long period of time before being released. But the most interesting aspect of the life cycle is what occurs in non-reptile hosts. Rodents are thought to be typical secondary hosts for the non-intestinal phase. Oocysts they ingest undergo excystation, sporozoites cross the gut wall and then become disseminated throughout the dermal tissues. Here, at least 2 asexual generations occur followed by sexual stages. The tissues around the face and neck become edematous (swollen) at this time (Figure 5D). Following fertilization, thin-walled oocysts are formed and 8 sporozoites develop within a membrane, but not a true sporocyst wall. These sporozoites rupture through the thin oocyst wall and enter macrophages and fibroblasts where they become dormant. These modified host cells with dormant sporozoites are termed caryocysts. When eaten by the appropriate snake, sporozoites are liberated from caryocysts and development proceeds in a manner thought to be identical to that which occurs when oocysts are ingested. Although at first glance this type of life cycle may seem unusually complicated, in reality, most developmental stages occurring within the mammalian host appear identical to those occurring in snakes.

Genera *Cyclospora* Schneider, 1881, *Eimeria* Schneider, 1875, and *Isospora* Schneider, 1881 (sensu stricto)

These 3 genera (Figures 6A–F) are considered together because they have mostly identical life cycles, as illustrated in the *Eimeria* cycle shown in Figure 6A. They differ only in the final morphology of their sporulated oocysts. After sporulation, eimerian oocysts have 4 sporocysts, each containing 2 sporozoites (Figures 6B and 6F–G), cyclosporan oocysts

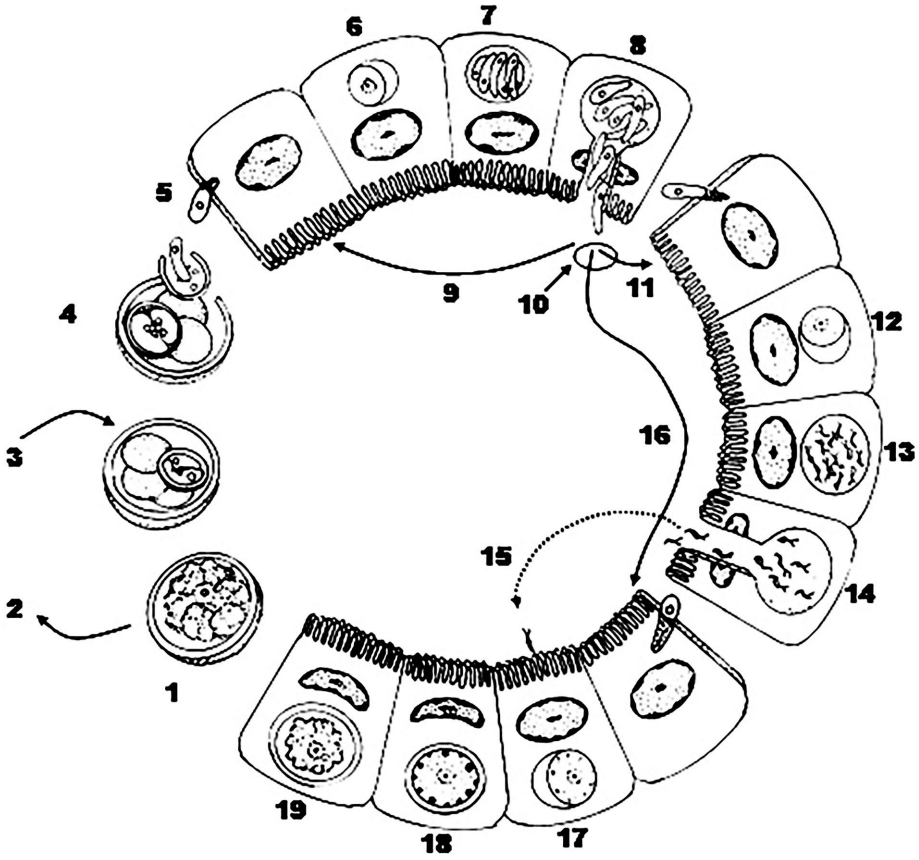
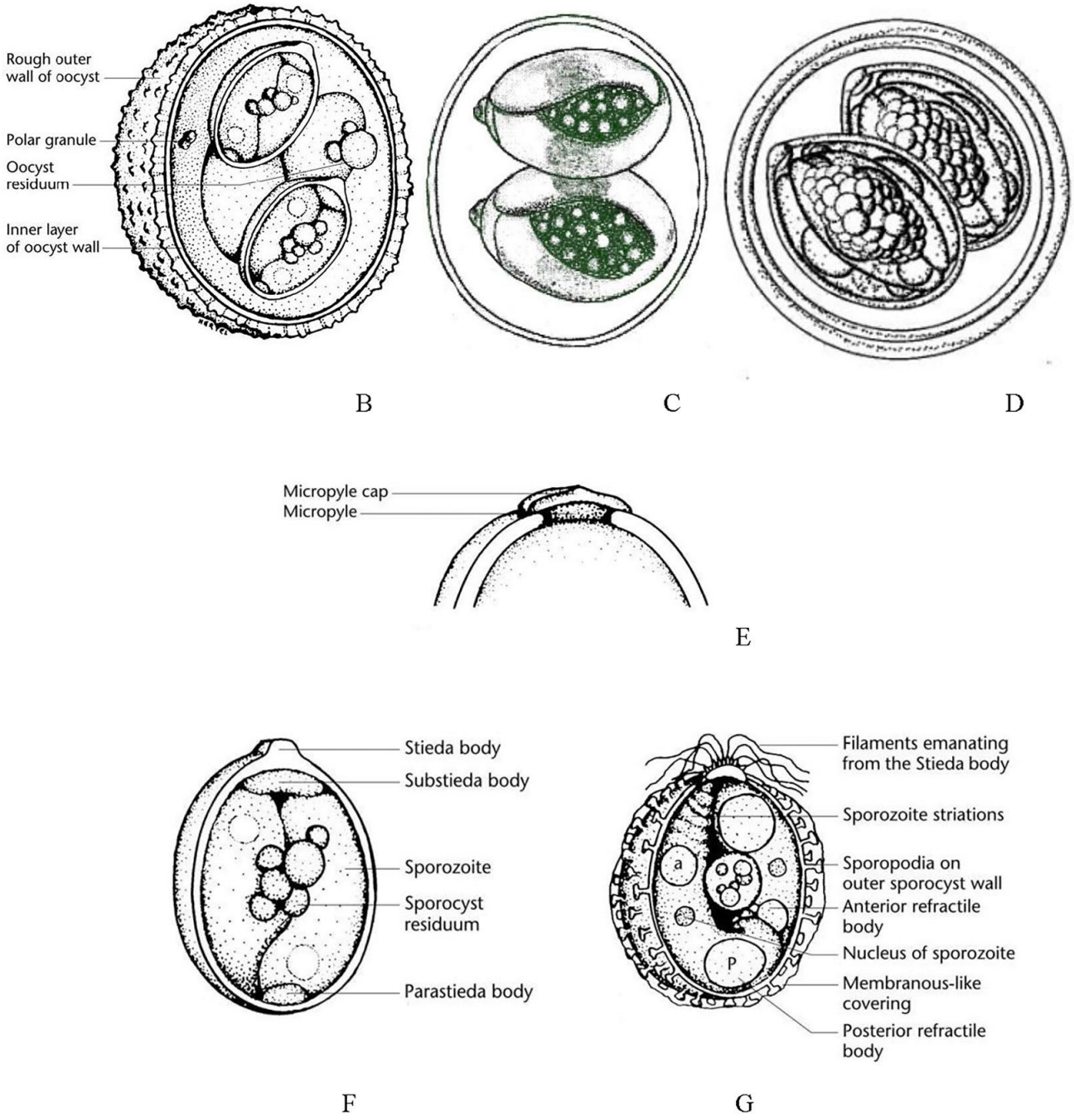


Figure 6A. Homoxenous life cycle of *Eimeria* species with a direct life cycle (*Cyclospora*, *Isospora* species have similar cycles). 1) Unsporulated oocyst leaves the host in its feces. 2) Oocyst needs molecular oxygen, moisture, and a temperature different than the host's body temperature to sporulate. During sporulation, 4 sporocysts, each with 2 sporozoites are formed. 3) Sporulated oocyst is infective to the next host. 4) Sporozoites are released from sporocysts/oocysts in host's gut. 5) Sporozoites penetrate host epithelial cells (6) then round up, enclosed in a parasitophorous vacuole to begin merogony. 7) Meront contains several to hundreds to thousands of merozoites. 8) Merozoites destroy host cell and may infect other cells (9) to produce more merogonous stages or (10) last generation of merozoites penetrate enterocytes to begin gamogony. 11) Microgametogony: the merozoite rounds up (12), many bi-flagellated microgametes are produced (13), rupture from their cell (14) and find a host cell with a developing macrogamont (15). 16) Macrogametogony: merozoite rounds up, producing a young macrogamete. After the microgamete penetrates the host cell (15) and fertilizes the macrogamete a young zygote is produced (17). Soon after, wall forming bodies (18) migrate to periphery of cell where they eventually coalesce to form the resistant oocyst wall; once wall is formed and the sporoplasm condenses, (19) the unsporulated oocyst ruptures from the host epithelial cell (1) to be discharged from the host in its feces. Source: Duszynski and Upton. License: CC BY-NC-SA 4.0.

have 2 sporocysts each containing 2 sporozoites (Figure 6C), and isosporan oocysts have 2 sporocysts each containing 4 sporozoites (Figure 6D). Numerous variations may be seen in different species on the surface structures of the oocyst and sporocyst walls (Figures 6B–G).

It is likely that an *Eimeria* species was 1 of the first protozoa visualized when Antonie van Leeuwenhoek saw what surely were oocysts of *Eimeria stiedai* Lindemann, 1895 in the bile of a rabbit in 1674. Since the oocyst is the stage that leaves the host, usually in the feces, it is the structure in the life cycle that is readily available to the veterinarian, wildlife biologist, or parasitologist who needs to identify the species without having to kill the host. As a result, about 98% of all *Eimeria*, *Isospora*, and *Cyclospora* species are known only from this 1 life cycle stage, the sporulated oocyst. *Eimeria*, with perhaps 2,000 named species to date, is the largest apicomplexan genus and may be the most speciose genus of all parasite genera (see Figures 7 and 8), and *Isospora* has about 250 named species; both have been reported in amphibians, reptiles, mammals, and birds and many *Eimeria* species (but not *Isospora*) have been reported in fishes. Fewer than 20 *Cyclospora* species have been named to date, most in mammals



Figures 6B–G. Line drawings of oocyst and sporocyst structures. B) Typical *Eimeria* oocyst, 4 sporocysts, each with 2 sporozoites. C) *Cyclospora* oocyst, 2 sporocysts, each with 2 sporozoites. D) *Isospora* oocyst, 2 sporocysts, each with 4 sporozoites. E) One end of an oocyst with a smooth outer surface and showing other possible structures, a micropyle and micropyle cap. F) Sporulated sporocyst showing major structural features including 2 sporozoites and the Stieda body/substieda body complex. G) Another sporulated sporocyst showing a variety of structural features, some of which may be present on sporocysts of different species. Source of all images: Duszynski and Upton, 2010. License: CC BY-NC-SA 4.0.

(insectivores, rodents, and primates) and a few in arthropods and reptiles. This genus is best known for 1 species, *Cyclospora cayetanensis* Ortega, Gilman and Sterling, 1994, a pathogenic coccidium transmitted by fecal contamination of food (fruits and vegetables) and water, that can cause diarrhea in humans and other primates.

The complete life cycle stages of a typical *Eimeria* species are shown in Figure 6A (see the figure legend for details) and

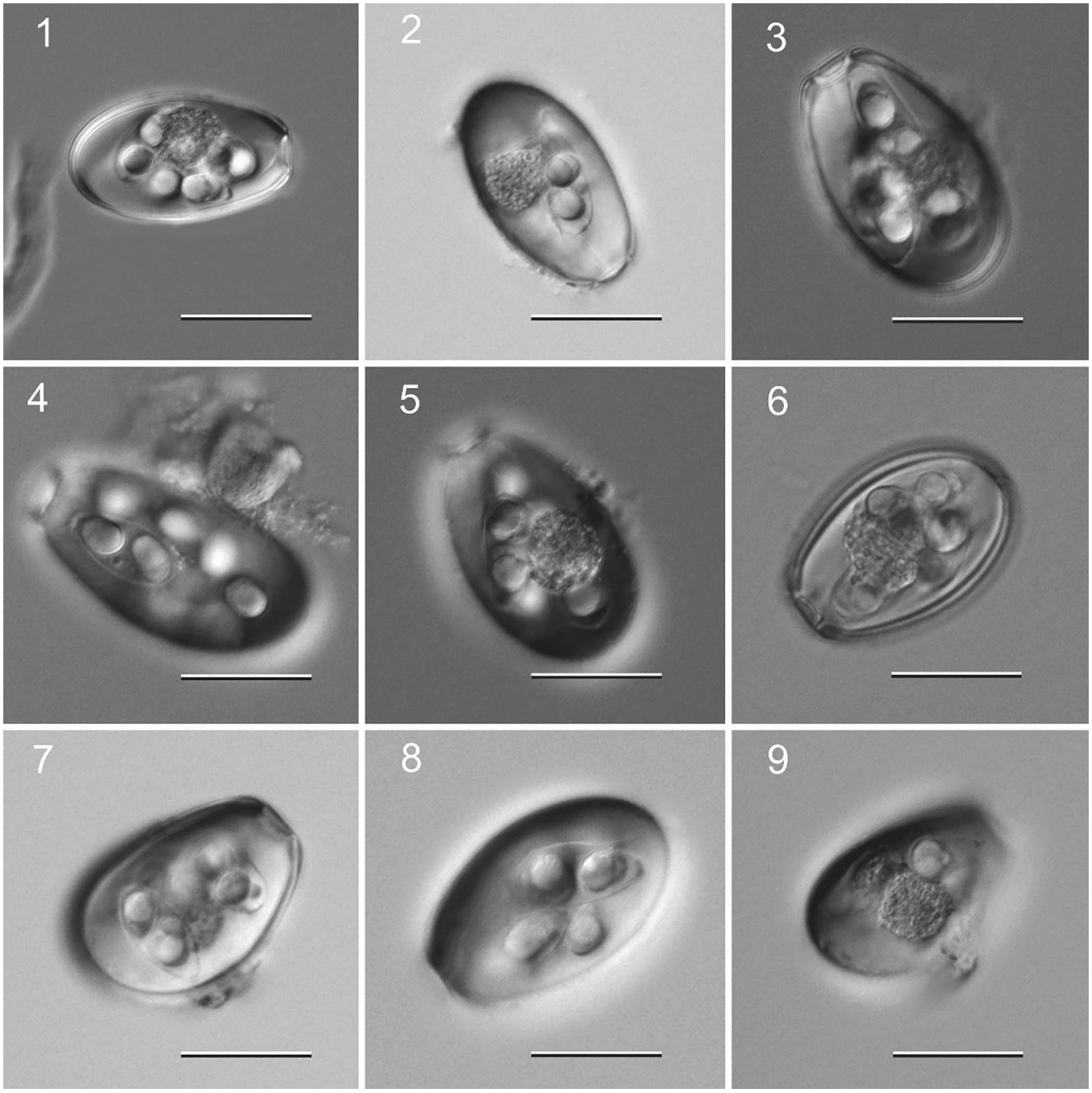


Figure 7. Examples of species of *Eimeria* from a Mongolian hare *Lepus tolai* from Mongolia. Scale bar = 25 μm. Source: S. L. Gardner, HWML. License: CC BY.

similar life histories are used by *Isospora* and *Cyclospora* species. To reiterate briefly, after a sporulated oocyst is ingested by a suitable host, sporozoites excyst and do so by both mechanical (via muscular contractions) and enzymatic (via trypsin or bile salts) digestive processes of the upper gastrointestinal tract in their host. These make the sporocyst and oocyst walls more permeable. Eventually, certain parts of each may be digested, or they may collapse or are broken, releasing their sporozoites so they can penetrate host epithelial cells. Invasion of the host cell is complicated, involving a sequential series of steps including recognition of a host cell, attachment to surface components, formation of a tight junction, entry into the cell (facilitated by organelles of the apical complex), and formation of a parasitophorous vacuole (PV) around the sporozoite (Sam-Yellowe, 1996). Inside its PV, the sporozoite initiates merogony (that is, asexual multiple fission).

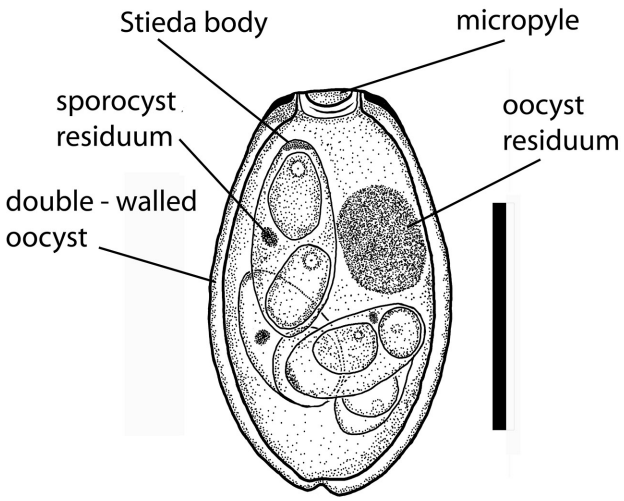


Figure 8. An oocyst of *Eimeria gobiensis* from a Mongolian hare *Lepus tolai* in Mongolia. Source: S. L. Gardner, HWML, 2009. License: CC BY.

During merogony, as few as 2, or up to as many as 100,000, merozoites may be formed by each sporozoite, depending on the species. Once mature, merozoites rupture the host cell, each seeking to penetrate a new epithelial cell to begin merogony again. It is believed that each species is genetically programmed for a specific number of merogonous generations. This was first demonstrated by Levine (1940). In this classic paper, he transferred merozoites of *Eimeria necatrix* from the intestine of 1 chicken to a second, coccidia-free chicken and showed that the time required for development of oocysts in the second bird was equal to that required in a single host, thus, showing that the length of the life cycle was determined not by increasing resistance of the host, but was inherent in each species of *Eimeria*. For the few coccidia species of which we know the actual number of asexual generations, it most often varies from 2 to 4 generations. Whatever the number, tremendous biological magnification of the parasite results from these developmental stages.

When the last generation of merozoites enter host epithelial cells, they develop not into additional meronts, but into gamonts. The vast majority develop into macrogametocytes (macrogamonts) to form uninucleate macrogametes, whereas the remaining merozoites develop into microgametocytes, each of which will undergo multiple fissions to produce thousands of motile, flagellated microgametes, but the precise mechanism that regulates if and when a merozoite will become a macrogamete or microgamete is unknown. Microgametes all are similar in structure with an elongate nucleus, an equally elongate mitochondrion, and 2 or 3 flagella (Scholtyseck, 1979). The nucleus occupies most of the space in the microgamete, which averages 4–7 mm-long. The elongate mitochondrion, about 2–5 mm-long, lies closely adjacent to and often in a groove of the nucleus. When they are mature, microgametes exit their host cell to seek out and penetrate cells with a mature macrogamete, but how microgametes find cells with developed macrogametes inside them, and details of the fertilization process, are unknown and warrant further study. When fertilization does occur, the diploid (2n) condition is restored. Thus, infections with these 3 genera are self-limiting as asexual reproduction does not continue indefinitely.

Family Sarcocystidae Poche, 1913

A second major family in the Eimeriorina, Sarcocystidae Poche, 1913, has 3 subfamilies, Cystoisosporinae Frenkel et al., 1987, Sarcocystinae Poche, 1913, and Toxoplasmatinae Biocca, 1957. All have *Isospora*-like oocysts in their life cycles with 2 sporocysts, each containing 4 sporozoites, but none of the sporocysts ever have a Stieda body. Instead, their sporocysts have longitudinal sutures that divide the surface into 4 or more plates.

Subfamily Cystoisosporinae Frenkel et al., 1987.

Frenkel et al. (1987) noted, “How we classify the heretofore unthought of cycles and stages is a scientific problem of taxonomy rather than of nomenclature” (page 250). Their new taxonomic ideas on these genera with heteroxenous life cycles reflects on the reproductive and transmission strategies of the parasites while maintaining the nomenclature. For this reason, they created 3 separate taxonomic concepts (subfamilies) for the isosporid coccidia without Stieda bodies in the interest of stability, uniqueness, and distinction.

Genus *Cystoisospora* Frenkel, 1977. Frenkel (1977, pages 620 and 625) erected the genus *Cystoisospora* to include those mammalian *Isospora* species with no Stieda body complex in their sporocysts, and with the ability to produce unique monozoic tissue cysts (MZTC) in intermediate or paratenic hosts, and these MZTC stages are a defining character of *Cystoisospora* species. Earlier, Frenkel and Dubey (1972) discovered the occurrence of tissue cyst stages of 2 intestinal coccidia of cats, *C. felis* and *C. rivolta*, in rodent paratenic hosts. They (1972) and others (Dubey, 1975; 1978a; 1978b; Rommel and Zielasko, 1981) also demonstrated that extraintestinal (EIN) stages can occur in the tissues (mesenteric lymph nodes, liver, spleen, lungs, brain, and musculature) of cats and dogs (which are definitive hosts) when fed sporulated oocysts of *C. felis* and *C. rivolta*, or *C. canis*, respectively. When either sporulated oocysts or infected intermediate hosts are ingested, these

parasites undergo merogony and gamogony in the intestinal epithelial cells of the carnivore definitive host and, ultimately, they discharge **un**sporulated oocysts with relatively thick walls (for example, *C. felis* and *C. rivolta* of felids; *C. canis, C. ohioensis*, and *C. vulpina* of canids). Thus, oocysts of *Cystoisospora* species look identical to *Isospora* species except for their ability to infect additional host species (Fayer and Dubey, 1987).

Subfamily Sarcocystinae Poche, 1913.

This is the second subfamily within the Sarcocystidae Poche, 1913 and contains 2 genera (*Sarcocystis* and *Frenkelia*). Votýpka et al. (1998), Modrý et al. (2004), and others consider *Sarcocystis* and *Frenkelia* as synonyms.

Genus *Sarcocystis* Lancaster, 1882. Miescher (1843) was the first to see what he called milky white threads (which were actually sarcocysts) in the skeletal muscles of a house mouse in Switzerland, and Huet (1882) saw the first sarcocysts in the muscles of a carnivore, a sea lion that died in the Jardin des Plantes de Paris, France. Lankester (1882) introduced the genus name for these Miescher's tubules to reflect what he saw, muscle (in Greek, **sarco** means flesh or muscle) and cyst (in Greek, **cyst** means bladder or bag), and

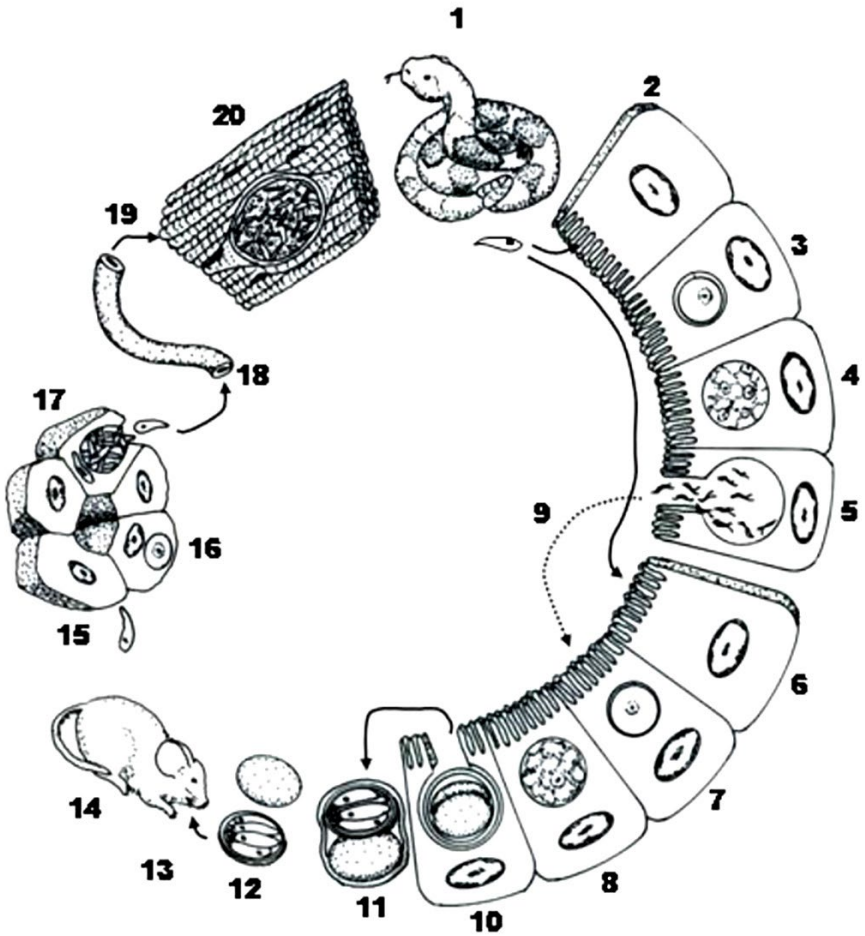


Figure 9. Typical life cycle of a *Sarcocystis* species with its obligate indirect life cycle. 1) Definitive host ingests infected prey items with sarcocysts in their tissues, bradyzoites are released and penetrate enterocytes of small intestine (2) where they develop directly into micro- (3–5) or macrogametocytes (6–8). After fertilization (5–7), sporogony occurs (8) in the lamina propria and sporulated oocysts are slowly released into the gut lumen fully formed and infective. (10) Oocyst wall is thin, it often ruptures during transit down the intestinal tract releasing 2 sporocysts in the feces, each with 4 sporozoites, rather than intact *Isospora*-type oocysts. 11) When oocysts and/or sporocysts are ingested by intermediate hosts, excystation occurs in the small intestine, sporozoites penetrate gut wall and enter a variety of extraintestinal tissues (12–14). 15–18) Precystic merogony usually occurs in tissues and merozoites from the last generation to enter the blood and are carried to striated muscles throughout body where they become bradyzoites and initiate sarcocyst formation. 19) Sarcocysts, with thousands of infective bradyzoites (20), are infective to the definitive host when it ingests an infected prey animal. Source: Duszynski and Upton, 2010. License: CC BY-NC-SA 4.0.

Blanchard (1885) named the organism *Miescheria hueti*. Finally, Labbé (1899) transferred this parasite to the genus *Sarcocystis*. The seminal work by Fayer (1970; 1972) first reported the transformation of bradyzoites from muscle cysts in grackles (*Quiscalus quiscula*) into gametocytes and oocysts in cell culture, and this was soon followed by Rommel et al. (1972) who described the shedding of sporulated sporocysts from cats after they ingested sarcocyst-infected mutton (also known as *Sarcocystis tenella*). Thus, the life cycle of all *Sarcocystis* species is now known to be an obligate, indirect cycle in which the definitive host is a carnivore in which only gametogony occurs, with the release of thin-walled sporulated oocysts or individual infective sporocysts; these stages must be ingested by a suitable intermediate host, in which tissue sarcocysts develop, and only these sarcocysts are infective for the definitive host (but not oocysts/sporocysts) (Figure 9).

Dubey et al. (2015) published an extensive treatise on *Sarcocystis* species in humans and other animals and listed 195 names as valid (Table 24.1 in Dubey et al., 2015), 49 *Sarcocystis* species names as invalid (Table 24.2 in Dubey et al., 2015), and 83 names (*Sarcocystis* sp.) that have never received a binomial. Students, and all interested readers, in all disciplines, should use these references when interested in maximizing *Sarcocystis* species data for any particular host species group.

Box 4. *Sarcocystis muris* Transmission — Learn More

Smith and Frenkel (1978) found sarcocysts in skeletal muscles of some lab mice housed in the same room as cats that had shed sporulated sporocysts of *Sarcocystis muris*. They noted that cat feces never came in proximity with mouse cages, but they saw German cockroaches (*Blatella germanica*) in the same room from time to time. To assess the role of *B. germanica* and the American cockroach (*Periplaneta americana*) in transmission, cockroaches were exposed to cat feces that contained oocysts/sporocysts of *S. muris, Isospora felis,* and *Toxoplasma gondii*. They found that *S. muris* sporocysts, which remained infectious in cat feces for at least 20 days, were transmitted to mice by *P. americana* for at least 20 days, and by *B. germanica* for 5 days post-exposure to infected cat feces.

Subfamily Toxoplasmatinae Biocca, 1957.

There are 3 important genera within this third subfamily, Toxoplasmatinae, which need to be mentioned. All of them have somewhat unusual complicated life histories and all of them have *Isospora*-type oocysts, but they are small and their sporocysts do not have Stieda bodies. An overview of each genus is covered below.

Genus *Besnoitia* Henry, 1913. Darling (1910), in Panama, found unusual cysts in an opossum, *Didelphis marsupialis*, and thought the parasite was a species of *Sarcocystis*, even though he expressed concern with some of the features in his cysts from the defining characteristics of the genus. Besnoit and Robin (1912), in France, found a protozoan that caused cutaneous and internal lesions in cattle associated with subspheroidal cysts. They also tentatively referred to their organism as *Sarcocystis*, but did not propose a binomial. Marotel (1912), unaware of Darling's (1910) paper, discussed Besnoit and Robin's (1912) work, and wrote, "Nothing similar has been found in animals ... and this is why I propose to designate their parasite with the name *Sarcocystis besnoiti*" (Jellison, 1956). The next year, Henry (1913) reexamined the characteristics of the organism, and the nomenclature assigned to it, and used the genus name *Besnoitia*.

Besnoitia species are obligatory heteroxenous coccidia, similar to those of *Sarcocystis* species, but they differ from *Sarcocystis* in 2 unique ways: 1) Oocysts are shed unsporulated by their definitive hosts and have relatively thick walls; and 2) these species can be successfully propagated asexually by mechanical transmission from intermediate host to intermediate host by blood-sucking arthropods. Their life cycles are similar to those of *Sarcocystis* species because the completion of the sexual cycle in the definitive host is dependent upon ingestion of tissue cysts from a suitable intermediate host—that is, the ability of oocysts to initiate gametogenesis in the definitive host also has been lost. Other details of what little is known about the life cycle of various *Besnoitia* species are summarized elsewhere (Leighton and Gajadhar, 2001; Dubey et al., 2003; Houk et al., 2011; Charles et al., 2011; and Duszynski and Couch, 2013). *Besnoitia* is the fifth apicomplexan to be a mammalian tissue parasite, along with *Cystoisospora, Hammondia, Sarcocystis,* and *Toxoplasma*. There are now approximately 10 valid species in this genus; the definitive host, which is a carnivore, is only known for 4 or 5 of these species and it is the domestic cat (*Felis silvestris catus*).

Genus *Neospora* Dubey et al., 1988. In 1984, a neuromuscular syndrome in dogs that simulated toxoplasmosis was documented by 3 Norwegian veterinarians (Bjerkås et al., 1984), who reported a protozoan causing severe encephalomyelitis in 6 Norwegian pups, but which had no antibodies to *Toxoplasma gondii*. All dogs originated from 3 litters from a single Boxer female. The pups appeared healthy until 2 months old. Five of these pups had neurological signs for several months, and all 6 were examined at necropsy and diagnosed with encephalitis and myositis with protozoa found in the lesions, including numerous tachyzoites and a few tissue cysts in their brains. Ultrastructural examination of tachyzoites showed them to be similar to those of *T. gondii*, but with more rhoptries. This confirmed the vertical transmission of this new, unnamed protozoan parasite.

Dubey et al. (1988) examined tissue sections and case histories from all dogs and cats that had died of a *Toxoplasma gondii*-like illness from 1952 to 1987 and were archived at the Angell Memorial Animal Hospital (AMAH), Boston, Massachusetts, the largest hospital for dogs and cats in the United States, which keeps meticulous records of pathology cases. Together, they examined thousands of slides from dogs and cats, and concluded that the syndrome recognized by Bjerkås et al. (1984) was not toxoplasmosis (see the review in Dubey et al., 2017). The records also showed that, in addition to neuromuscular clinical signs, dogs suffered severe disease involving the heart, lungs, liver, and the skin. Dubey et al. (1988) found a similar parasite in formalin-fixed tissues from 10 dogs in the United States, named a new genus, *Neospora*. *Neospora caninum* Dubey et al., 1988 later became the type species.

A decade later, McAllister et al. (1998) firmly established dogs as the definitive host of *Neospora caninum* and their genus definition included: 1) Tissue cysts in several cell types, but primarily in the neural tissues; 2) a tissue cyst wall up to 4 μm thick, much thicker than *Toxoplasma gondii* tissue cysts (~ 0.5 μm); 3) numerous bradyzoites, not separated by septa; 4) tachyzoites with numerous electron-dense rhoptries, some posterior to the nucleus; 5) canids (dog, coyote, and wolf) as definitive hosts and many intermediate hosts, including dogs, cattle, horses, goats, deer, water buffaloes, coyotes, red foxes, and camels (see also Dubey, 1999; Lindsay and Dubey, 2000); 6) tachyzoites and tissue cysts in both intermediate and definitive hosts; 7) oocysts excreted unsporulated; 8) antibodies to *T. gondii* not present in infected dogs and the parasites not reacting to *T. gondii* antibodies in immunohistochemical tests; 9) transmission by carnivorism, transplacental and fecal; and 10) tachyzoites, tissue cysts, and oocysts all infectious to both intermediate and definitive hosts.

Box 5. Neosporosis — Learn More

Considerable progress in understanding the biology of neosporosis has been made in the last 30+ years, re-sulting in more than 2,000 scientific publications! For the interested reader, Dubey et al. (2017) have written a comprehensive, well-organized, easily-read book on this subject.

Genus *Toxoplasma* Nicolle and Manceaux, 1909. There may be several *Toxoplasma* species in poikilotherms (Duszynski and Upton, 2010), but most parasitologists who work in this area believe there is only 1 species, *T. gondii*, in mammals, and it has worldwide distribution. Prior to the early 1970s it was thought that *T. gondii* might be transmitted by blood sucking arthropods, but it is now known that felids are the definitive host. Clinical toxoplasmosis has been reported in virtually all species of warm-blooded animals, including humans, and domestic and wild animals (Dubey and Beattie, 1988; Dubey, 2010). In fact, *T. gondii* may be the most ubiquitous parasite on Earth because it can be transmitted directly (fecal/oral, including using arthropods as mechanical vectors), transplacentally, and by carnivorism (see Chapter 8, p. 133–140 in Duszynski, 2016 for a brief review).

Toxoplasma gondii has an indirect life cycle with only felids serving as definitive hosts in which the parasite goes through both asexual and sexual endogenous development in intestinal epithelial cells. All other vertebrate animals that ingest sporulated oocysts are susceptible to infection but, in them, *T. gondii* forms cysts in cells of virtually any tissue in the body. If these tissue cysts are eaten by another omnivore or non-felid carnivore, the process can be repeated, with the development of tissue cysts in the new host. When cats consume a host animal harboring mature tissue cysts, endogenous development in the gut can be initiated (depending upon the cat's immune status to *T. gondii* from a previous infection), and/or bradyzoites from the ingested cysts can go on to develop in the tissues of the cat, too.

Dubey and Frenkel (1972) outlined the sequence of events in the epithelial cells of cats inoculated orally with tissue cysts of *Toxoplasma gondii* and found 5 new structural stages they designated as types A–D. Interestingly, the feeding of each of the 3 principal *T. gondii* stages to cats results in different prepatent periods. If chronically-infected mice

(characterized by older tissue cysts with bradyzoites) are fed to cats, oocysts can be found in cat feces 3–5 days post-infection (dpi). Cats fed acutely-infected mice (characterized by young tissue cysts with tachyzoites) won't shed unsporulated oocysts until 5–10 dpi, and cats fed sporulated oocysts usually do not begin to shed oocysts until at least 20–24 dpi.

The mechanisms by which *Toxoplasma gondii* is transmitted in nature to maintain its ubiquity as an infectious agent still are not completely understood because they are so highly varied. Insects in nature can become infected and, if ingested by mammals or birds, insects may be important transport or paratenic hosts. Wallace (1971) demonstrated the potential of both *Musca domestica* (common house fly) and *Chrysomya megacephala* (latrine fly) to be able to transmit sporulated oocysts of *T. gondii* for at least 24 and 48 hours, respectively, and *Periplaneta americana* (American cockroach) and *Rhyparobia maderae* (Madeira cockroach) for up to 12 days post-infection. However, to be of practical interest, it needed to be determined that some of the more prevalent cockroaches were prone to ingest cat feces. Chinchilla and Ruiz (1976) worked with 3 of the most common cockroaches in Costa Rica, *P. americana, P. australasiae,* and *R. maderae*, by experimentally showing that both *Periplaneta* species ate cat feces even in the presence of common foods (for example, dough, sugar, bread, cheese) found in most Costa Rican homes, and that *R. maderae* showed the greatest tendency to ingest cat feces. Their results suggest that these insects are potential transport hosts for oocysts of *T. gondii* in cat feces. They also noted that these 3 cockroach species are the most common in city markets, where cats also abound. Also, Smith and Frenkel (1978) found *P. americana* and, to a lesser extent, German cockroaches (*Blatella germanica*), transmitted *T. gondii* oocysts to mice for up to 10 days post-exposure to infected cat feces.

Oocysts of *Toxoplasma gondii* also can last a long time in the external environment. Frenkel and Dubey (1973) determined that sporulated oocysts suffer little attrition after constant or intermittent freezing at −6 °C, but greater attrition at −21 °C, and that sporulated oocysts survive −20 °C for 28 days, indicating that freezing weather alone does not eliminate oocyst infectivity from soil contaminated by cat feces. Frenkel et al. (1975) looked at the effects of freezing and soil storage in Costa Rica and Kansas, United States. In Costa Rica, infectivity persisted for 1 year in 3 shaded sites, 2 moist sites, and 1 relatively dry site in the soil, and in Kansas infectivity lasted up to 18 months, including 2 winters. Frenkel et al. (1975) also recovered oocysts from the surface of 1 *Musca*, several soil isopods, and earthworms. Dubey (1998) looked at the survival of sporulated *T. gondii* oocysts under defined temperatures, and then tested their infectivity by mouse bioassay. There was no marked loss of infectivity of oocysts stored at 10-, 15-, 20-, and 25 °C for 200 days; oocysts stored at 35 °C were infective for 32, but not at 62 days, those at 40 °C were infective for 9, but not 28 days, those at 45 °C were infective for 1 day, but not for 2 days. Sporulated oocysts remained infective up to 54 months at 4 °C, and no loss of infectivity was seen in oocysts stored for 106 days at −5 °C and −10 °C, and for 13 months at 0 °C.

There have been thousands of surveys around the world looking for oocysts in cat feces and testing blood for antibodies in a variety of in vitro tests, and inspecting tissues for cysts in many other vertebrates, including many carnivores. Dubey (1976) pointed out that even though > 60% of cats in the United States and elsewhere have antibodies to *Toxoplasma gondii*, only about 1% or less are found to be shedding unsporulated oocysts at any given time. Weiss and Kim (2007) contributed a definitive textbook on the perspectives and methods of *T. gondii* as a model apicomplexan.

Cryptosporididae Tyzzer, 1907

The taxonomy of this group has changed considerably since it was discovered by Tyzzer (1907; 1910) because it possesses features of both coccidians and gregarines. It was initially classified with the Coccidia, but it was found later to be phylogenetically more closely related to Gregarinasina (Carreno et al., 1999; Barta and Thompson, 2006; Kuo et al., 2008). Currently, it is a distinct group of the Conoidasida, on equal status with the Coccidia and the Gregarinasina (Adl et al., 2012).

Genus *Cryptosporidium* Tyzzer, 1907

Formerly, *Cryptosporidium* was thought to be a monospecific genus (Tzipori et al., 1980; Tzipori and Campbell, 1981) because of its presumed lack of host specificity, nearly identical life cycle developmental stages (both exogenous and endogenous), and their shared antigenicity (see Figure 8). However, with the advent of gene sequencing and other molecular innovations that tease apart subtle genetic differences, it is now believed there may be at least 30 valid species, and > 50 genotypes, many of which may be mostly adapted to a narrow spectrum of hosts (Lucio-Forster et al., 2010; Osman et al., 2015; Lihua Xiao, personal communication). However, this area of study is still a work in progress and no definitive documentation exists yet regarding the exact number of *Cryptosporidium* species (Plutzer and Karanis, 2009; Fayer et al., 2010). Many isolates have been classified as "genotypes," without species definitions (Fayer, 2010) or binomials, and may simply represent cryptic species.

Cryptosporidium species are obligate, monoxenous, intracellular, but extracytoplasmic, parasites. They have been found to infect a wide variety of vertebrate species worldwide, including humans, their domestic food and companion animals, and many species of wild and laboratory animals. Since recognition of the seeming ubiquity of *Cryptosporidium* oocysts in host feces, searching for them has historically followed 2 paths. First, their oocysts are intentionally sought out in cases of chronic or acute clinical illness, especially in our domesticated and companion animals. Second, general, non-invasive surveys of larger sample sizes of various vertebrate populations have been conducted worldwide to determine prevalence. But prevalence studies rely principally on morphology of the oocysts found, and therein lies the problem. Oocysts are so small, nondescript, difficult to find, and they lack in mensural characters such that they are virtually impossible to use to identify species. At present, fecal flotation, several staining procedures, and immunofluorescence assays of fecal samples are the most commonly-used laboratory techniques for diagnosing *Cryptosporidium*. Thus, light microscopy (LM) is routinely used for diagnostics; however, it does not allow the identification of species because of the morphological uniformity of the oocysts. Moreover, LM suffers from low sensitivity, because the oocysts: 1) May be shed in small numbers, often under detectable levels; 2) are translucent and small (~ 4–7 μm-wide); and 3) may be confused with yeasts, fungal spores, and/or other structures in fecal samples. Thus, examination using LM requires a trained technician because the oocysts may be overlooked easily, or may be misdiagnosed, and lead to false-positive diagnoses. Moreover, since oocysts are shed intermittently, 1 negative fecal exam may not necessarily mean that the host individual is not parasitized. Therefore, repeated fecal exams should be undertaken when possible.

Oocysts already are sporulated when passed, thus immediately infective. They remain infective in the environment for a long period of time, are resistant to most common disinfectants, and also are able to survive routine wastewater treatment (Fayer et al., 2000; Ryan and Power, 2012). The most common environmental and alimentary sources of *Cryptosporidium* are water treatment facilities, raw sewage discharge, especially into rivers, wells, ditches, and oceans, where molluscs, oysters, and vegetables become exposed (Meireles, 2010). When sporulated oocysts have been ingested by a suitable host (Figure 10), the infection is usually self-limiting in immunocompetent individuals, but may become acute leading to morbidity and mortality in immunocompromised ones. Therefore, *Cryptosporidium* species can and do have a great influence on public health.

Cryptosporidium Diagnosis and Genotyping

Due to their small size, intermittent shedding, and limited morphological variation, only molecular and immunological methods can begin to tease apart the subtle sequence or genetic differences between *Cryptosporidium* species and genotypes. Of particular relevance in evaluating, detecting, resolving, and differentiating the identity of *Cryptosporidium* species, the following diagnostic and genotyping methods are particularly useful: Polymerase chain reaction (PCR), real-time PCR, nested PCR-RFLP, IMS-qPCR, qPCR-MCA assay, enzyme immunoassays, and sequencing of specific genes or regions (Feng et al., 2009; Gao et al., 2013; Homem et al., 2012; Jiang and Xiao, 2003; Lalonde et al., 2013; Leoni et al., 2006; Lindergard et al., 2003; Silva et al., 2013; Xiao et al., 2004). In the case of *Cryptosporidium* species, several markers or loci are now commonly employed to determine species or genotype differences including, but not limited to, partial and full sequences of 18S rRNA, *Cryptosporidium* oocyst wall protein (COWP), 70 kDa heat shock protein (Hsp70), glycoprotein 60 (gp60), and actin genes, with partial 18S rRNA gene sequences being the most commonly used marker. Clearly, combining as many of these techniques as possible is much more sensitive in detecting *Cryptosporidium*-positive fecal samples (Morgan and Thompson, 1998; McGlade et al., 2003; Scorza et al., 2003; Fayer et al., 2006; others) than could be expected under only LM. However, a positive PCR does not provide information on the viability and infectivity of the pathogen. Thus, a combination of methods (LM, TEM, molecular detection, and immunological methods) is recommended and vital, especially in cases where only a few oocysts are present in the feces, or when any doubts are raised regarding the diagnosis, especially in the isolates involved in human outbreaks and/or epidemiological studies.

The diversity demonstrated by *Cryptosporidium* species is not surprising. Gregarines are ubiquitous, incredibly diverse parasites, with thousands of species so far described and a heterogeneity of life cycle patterns and developmental forms. The recognition of *Cryptosporidium* affinities with this group helps to explain the increasing numbers of novel genotypes that are being discovered and emphasizes that the specificity of environmental detection procedures for *Cryptosporidium* could be compromised by cross-reactivity with gregarine protozoa that are ubiquitous in freshwater environments (Bull et al., 1998; Hijjawi et al., 2002; Tenter et al., 2002).

A better understanding of the developmental biology of *Cryptosporidium* in its host can now be achieved by a more comparative approach with what is known of some higher gregarines. This applies to the parasite's relationship with

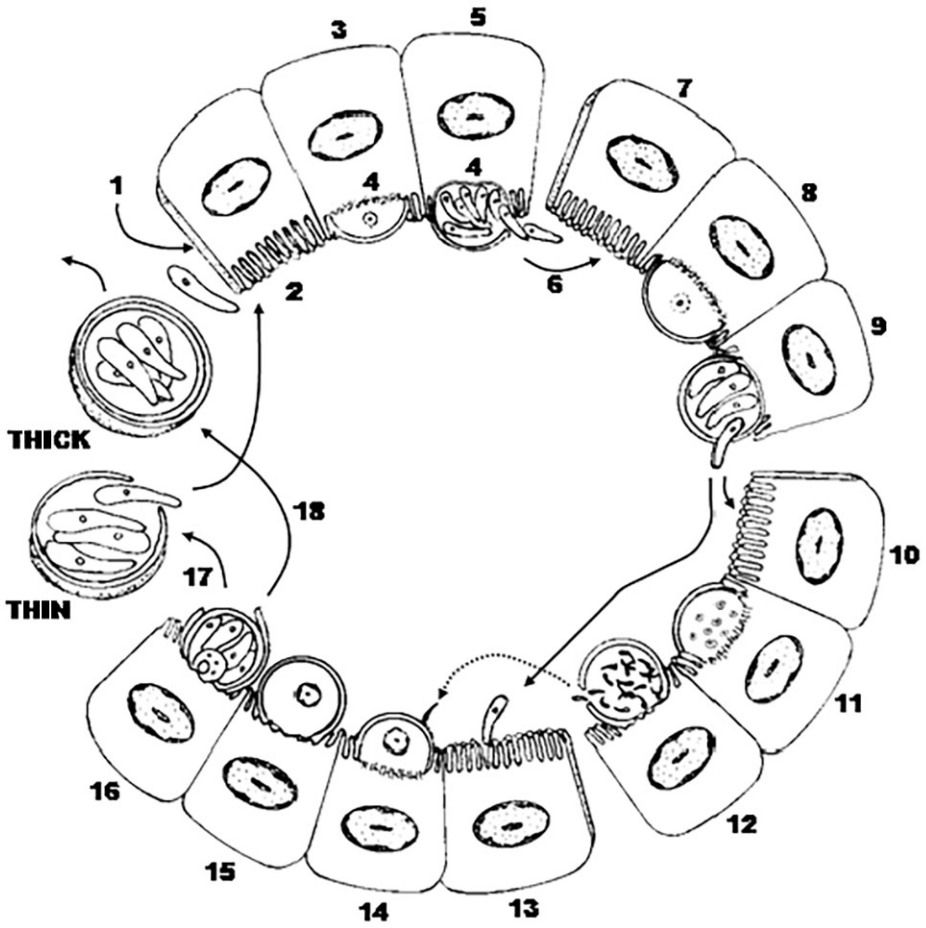


Figure 10. Direct life cycle of a *Cryptosporidium* species. 1) Ingestion of sporulated (thick-walled) oocyst (4 sporozoites) with contaminated food and/or water. 2) Sporozoites excyst from oocyst and penetrate the microvillus layer of epithelial cell and become enclosed by a thin layer of host cell cytoplasm and membranes (3). 4) A desmosome-like attachment organelle and folding of the parasite membranes develop at the interface between parasite and host cell cytoplasm. 5) Merogony forms 8 merozoites in Type I meront. 6) The meront ruptures the host cell releasing merozoites, which penetrate new host cells (7) forming Type II meronts (8). 9 and 10) Type II merozoites enter other epithelial cells to become microgametocytes (11) that undergo multiple fission (12) to produce ~16 non-flagellated microgametes. (13) Most Type II merozoites penetrate epithelial cells enlarging into a macrogametocyte/macrogamont to become a macrogamete (14). 15) Cells with macrogamonts are penetrated by microgametes which penetrate a macrogamete to form a zygote. 16) Sporogony occurs releasing sporulated oocysts into the environment of the intestinal lumen and the feces. About 20% of oocysts fail to form an oocyst wall (17) and only a series of membranes surround the sporozoites. Sporozoites from these thin-walled oocysts are thought to excyst within the gut and infect new epithelial cells (1 and 2). The remaining 80% of thick-walled oocysts exit the host in the feces to potentially contaminate food and water of future hosts. Source: Duszynski and Upton. License: CC BY-NC-SA 4.0.

its host cell and whether *Cryptosporidium*'s epimerite-like feeder organelle obtains nutrients in a way that is truly analogous to myzocytosis, as utilized by many gregarines, through which host cell contents are obtained. In this respect, it is interesting that the feeder organelle has been observed in extracellular stages in a biofilm environment and thus may be able to acquire nutrients in such a host cell-free environment (see Koh et al., 2014).

Discussion, Conclusions, and Difficulties of Working with Apicomplexa

Species Identifications

Accurate species identification is fundamental to every biological investigation. Taxonomists who work with the Coccidia face numerous challenges when defining new species because these parasites undergo a sequential series of

structural changes, both inside (endogenous) and outside (exogenous) their host species. Endogenous developmental stages (multiple stages of merogony, merozoites, micro- and macrogametocytes, developing zygotes/oocysts) exhibit sequential structural changes and to find and measure them requires killing the host. Sporulated oocysts outside the host have been studied the most, historically, because they are resistant to environmental extremes, can be collected by non-invasive means (fecal collection/preservation), and usually can be maintained for long periods of time. Unfortunately, however, oocysts have only a small suite of qualitative and structural characteristics that are quantifiable in the Eimeriidae, but especially in the Cryptosporididae. Generally, the identification of *Eimeria*, *Isospora*, *Caryospora*, and other species in the family is based primarily on their oocyst features without other supporting information (Jirků et al., 2009). Thus, to date, ~ 2,000 nominal species of these genera have been described, with ~ 98% of them identified only by their oocyst's morphology (Asmundsson et al., 2006; Ghimire, 2010). The morphology of oocyst structures both within and between host species can be quite diverse to the point that it becomes confusing and is sometimes difficult to distinguish species based entirely on morphological features. Thus, morphology alone is no longer sufficient to confidently identify many coccidian species, especially those in genera with very small oocysts and sporocysts. These identifications should be supplemented by multiple data sets with information collected from, but not limited to, site of sporulation (endogenous versus exogenous), information on the location and sizes of some or all of the endogenous developmental stages, and sequence data to conduct phylogenetic analyses that will allow the investigator to more robustly assign a parasite to a group, genus, or even species (for example, see Merino et al., 2008; 2009; 2010).

When Do Oocysts Become Sexual?

There is only limited information on sexual differentiation in endogenous and/or exogenous life cycle stages. Canning (1963), Klimes et al. (1972), and others (Jeffers, 1978; Cornelissen et al., 1984; Cornelissen and Overdulve, 1985) showed that merozoites were sexually differentiated. This may happen either between the sporozoite and the first generation meront/merozoites or the first generation merozoites could be sexually undifferentiated, and gene expression is responsible for the formation of either male or female type second generation meronts/merozoites. Cornelissen et al. (1984) called these merozoites macro- or microgamontoblasts. Microgamontoblast merozoites were reported to have only a few granules of polysaccharide reserves and their nuclei lack nucleoli, while those giving rise to macrogamonts had abundant coarse granules of polysaccharide and the nuclei each have a conspicuous nucleolus. Both gamontoblasts contained the haploid amount of DNA and none has been found to be synthesizing DNA.

There is no good evidence that fertilization of a macrogamete is a necessary stimulus to form the oocyst wall, but in oocysts which do sporulate, the zygote sporoplasm is the only stage to possess a diploid nucleus. During the first nuclear division of sporogony, chromosome reduction occurs in a single meiotic division, whereas 2 subsequent nuclear divisions within the zygote are thought to be mitotic. Thus, sporozoites in each sporocyst, as products of a meiosis, would be genetically identical; since infection with either a single sporocyst, or even a single sporozoite, produces viable oocysts in the right host, suggesting that sexual differentiation occurs at a stage in the life cycle later than sporogony. This makes the 2 sexually undifferentiated sporozoites in each sporocyst the basic unit of propagation. It is clearly the advantage of the parasite to remain sexually undifferentiated until it is well established in the host, thus avoiding the possibility of unsuccessful infections due to the loss of sporozoites of the opposite type (Lee et al., 1977).

One sporulated oocyst doesn't necessarily represent a population of genetically identical organisms because it may contain recombination characters from 2 different parental lines. Once ingested and the sporozoites are released, they penetrate epithelial cells and merogony begins. The question then arises, when and where does sexual differentiation occur? The sexuality of individual sporozoites was debated throughout the 1960s and 1970s, but sufficient work was done to indicate they most likely are bisexual (Haberkorn, 1970; Shirley and Millard, 1976; Jeffers, 1978; Cornelissen et al., 1984; Cornelissen and Overdulve, 1985). That is, sexual differentiation is influenced by environmental stimuli responsible for their expression, but the exact nature of exogenous stimuli is unclear. This demonstrates that true clones of *Eimeria* can be established only from individual sporozoites or sporocysts. If sex were determined by genetic factors which segregate during zygotic meiosis, individual sporocysts would contain sporozoites of like sex and would be incapable of producing a complete infection that could produce zygotes.

Oocyst Production

The time between when a suitable host ingests a sporulated oocyst and when oocysts leave that host in its feces is termed the **prepatent period**. During this interval, which can vary from 3 to 10 days (or more), no oocysts are found in the feces because only merogony and the beginning of gamogony are occurring in the host. The time interval during which oocysts are discharged from an infected host is

termed the **patent period** and lasts until all the fertilized and unfertilized macrogametes have been released from their host cells. Both time periods vary between host and coccidian species and are dependent on many factors including: Coccidian species, number of oocysts ingested, number of endogenous stages for that species, depth within the tissues where merogony, gamogony, and fertilization occur concurrent infection with other parasites, host age, nutritional and immune status, and other ecological and physiological factors that are not yet understood.

Once outside the host, the oocyst must sporulate in many species before it is infective to another suitable host. The presence of oxygen, moisture, shade (direct exposure to ultraviolet radiation—sunlight—will kill oocysts quickly) and, generally, a temperature less than the body temperature of the host, all are necessary for oocyst survival. If these conditions are met, complete sporulation occurs and the fully formed oocyst and sporocysts are resistant to environmental extremes, and the sporozoites therein are immediately infective to the next suitable hosts that may ingest them. Each oocyst has a suite of structural characters, unique to its species, that can help the experienced taxonomist distinguish one species from the next in many instances. Unfortunately, because this suite of characters is so small, sometimes sporulated oocysts from different host species look very nearly identical in size and structure and may not be easily or reliably differentiated by morphological features alone. In these instances, life history information (for example, tissue stages as in *Choleoeimeria*) and molecular techniques (such as gene sequencing followed by phylogenetic analysis) is necessary to assist in final identification of the parasite under scrutiny.

Survival of Oocysts

Our understanding of the survival of oocysts in the external environment and the mechanisms by which they reach an appropriate definitive host is minimal and requires additional study. Moisture, temperature, and direct exposure to sunlight all influence the ability of oocysts to sporulate in the external environment (or not), but the interactions of these and other factors (for example, mechanical vectors such as invertebrates) are not well understood. In general, oocysts sporulate more rapidly at higher temperatures and slower at lower temperatures; exposure to temperatures less than 10 ºC or greater than 50 ºC is lethal to unsporulated oocysts. Between these extremes, the sporulation of oocysts in a field-collected fecal sample is dependent on at least the following factors: 1) The parasite species, 2) the time and temperature between collection and arrival of the sample at the laboratory, 3) the medium in which the sample was stored, 4) the amount of molecular oxygen available to the stored oocysts, and 5) the concentration of oocysts in the sample. Under optimal laboratory conditions, sporulation of oocysts from mammals occurs best between 20 °C and 25 °C, but this will vary among vertebrate classes (Duszynski and Wilber, 1997). Interestingly, a few oocysts of some *Eimeria* species (normally, 4 sporocysts each with 2 sporozoites) can be induced to change into *Isospora*-like oocysts (2 sporocysts each with 4 sporozoites) when fresh, unsporulated oocysts are first heated to 50 ºC for 30–60 seconds before incubation at 25 °C for a week (Matsui et al., 1989).

Once sporulated, oocysts of some species remain viable and infective in 2% aqueous potassium dichromate (kills bacteria, prevents putrification) at 4–5 ºC for up to 24 years (Williams et al., 2010)! In their natural external environment, oocysts remain viable and infective from as little as 49 days up to 86 weeks, dependent upon the species and the interplay of abiotic and biotic environmental parameters.

Other Means of Transmission?

The role that naturally occurring soil (for example, mites, ticks, earthworms, and so on) or household organisms (such as house flies and cockroaches) can serve as mechanical vectors has been little studied, but it is known that in many instances invertebrates can be important contributors to the continuation of coccidian life cycles. In *Hepatozoon* species we know that many invertebrate species (such as mites, ticks, and so on) serve as the definitive host while a vertebrate becomes the intermediate host, and gamonts of the parasite in its red blood cells must be ingested by the intermediate host for the cycle to complete. We know that *Besnoitia* species can be propagated asexually by mechanical transmission from intermediate host to intermediate host by blood sucking arthropods. Cockroaches are known to transmit oocysts/sporocysts of *Sarcocystis* species to mice in which sarcocysts can and/or will form. Goodwin and Waltman (1996) demonstrated that darkling beetles (*Alphitobius diaperimus*) could transmit sporulated oocysts of *Eimeria* species to chicks inoculated with beetle homogenates (also see Markus, 1974; 1980; Clubb and Frenkel, 1992).

It has been demonstrated experimentally that at least a few bird and mammalian *Eimeria* may form extraintestinal tissue stages (Carpenter, 1993; Mottalei et al., 1992). Apparently, sporozoites excyst from oocysts ingested by these hosts, infect cells in various places in the body and become dormant. The infected host may or may not be the ‘normal’ host for that *Eimeria* species; if the host with such tissue stages is eaten by the appropriate host, these dormant sporozoites become active, infect enterocytes (which are intestinal epithelial cells) and initiate their typical life cycle. It is not known if such a cycle is functional in natural

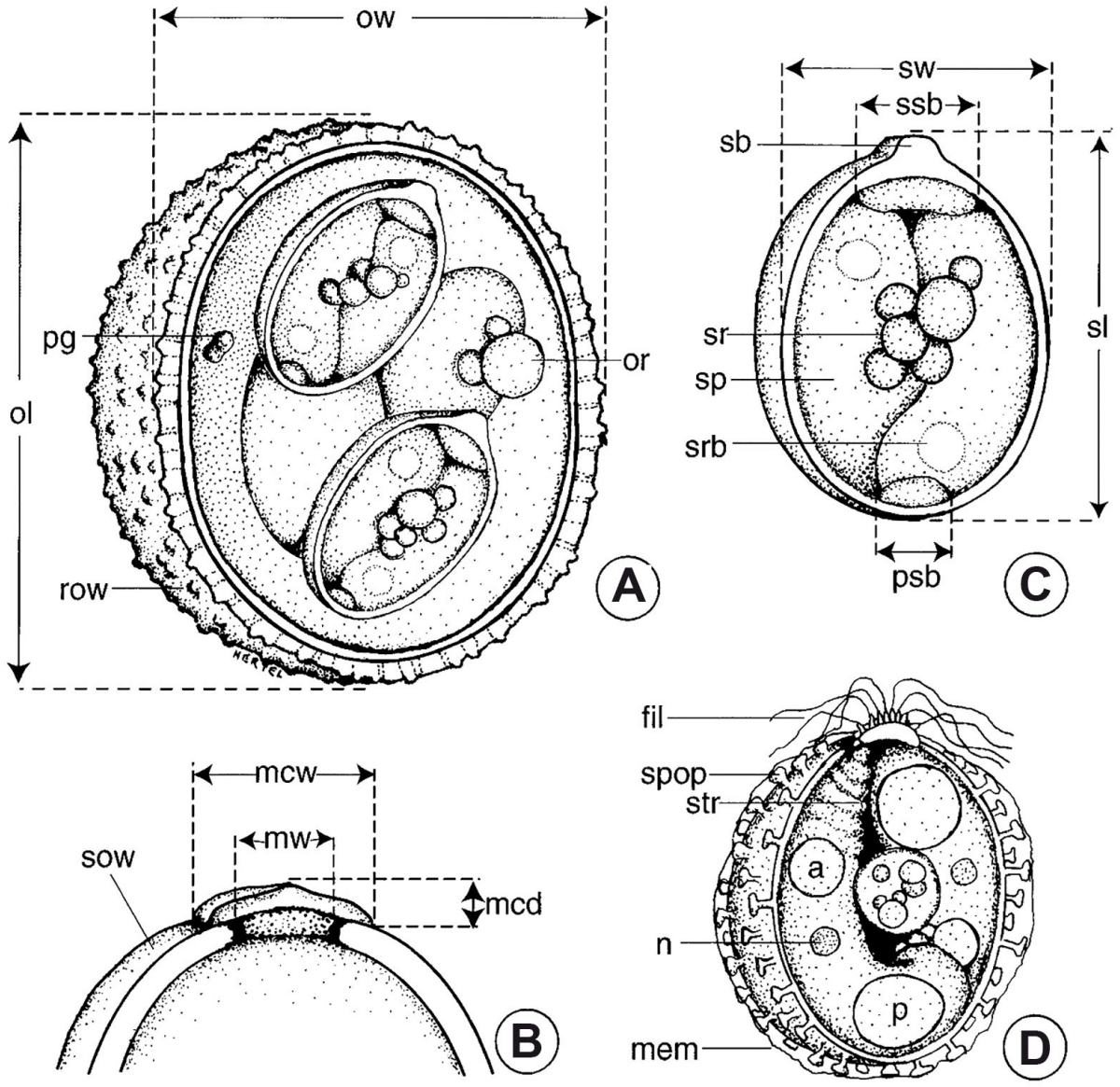


Figure 11A–D. Line drawings of the parts of sporulated oocysts (Eimeriidae: *Eimeria*, *Isospora*, et al.) that should be measured and carefully documented when submitting a new species description for publication. A) Sporulated oocyst of an *Eimeria* sp., drawn in optical cross section, showing essential structural parts that should be measured/documented in the species description: **ow**, oocyst width, measure the widest part when the oocyst is in good optical cross section under oil immersion; **ol**, oocyst length; **pg**, polar granule, note shape and size; **or**, oocyst residuum, note shape, structure, size, and whether or not it may be membrane bounded; **row**, rough outer wall, note this feature, if present, as well as its thickness relative to the inner wall (if present). B) The top of an oocyst that has a micropyle, micropyle cap, and a smooth, 1-layered wall: **sow**, smooth outer wall; **mw**, width of the micropyle; **mcw**, width of the micropyle cap; **mcd**, depth (= height) of the micropyle cap. C) Composite sporulated sporocyst (hypothetical) from an oocyst of *Eimeria* sp., drawn in optical cross section, and enlarged to show detail: **sw**, sporocyst width, measure the widest part when the sporocyst is in optical cross section under oil immersion; **sl**, sporocyst length; **sb**, Stieda body; **ssb**, substieda body, measure width and note relationship to sb (for example, 2 × wider); **psb**, parastieda body, measure width and height (if possible); **sr**, sporocyst residuum, note shape, structure, size, and whether or not it may be membrane bounded; **sp**, sporozoite, note any peculiar or unique features; **srb**, sporozoite refractile body, note size, number, and relative locations in sp. D) Composite sporulated sporocyst (hypothetical) showing a number of unique structural features that may be present in/on the sporocysts/sporozoites of certain eimeriid species: **fil**, filaments emanating from the area of the Stieda body; **spop**, sporopodia extending from the outer surface of sporocyst wall; **mem**, membranous-like covering sometimes associated with sporopodia; **n**, a nucleus sometimes is visible within sporozoite; **str**, sporozoites sometimes have striations at their anterior end; although some sporozoites have only 1 refractile body (Figure 9C), others have both anterior (a) and posterior (p) refractile bodies as shown here. Source: Original by L. A. Hertel; adapted from Duszynski and Wilber, 1997.

communities. And, of course, some *Caryospora* species are facultatively heteroxenous. *Cystoisospora*, *Besnoitia*, and *Toxoplasma* species form tissue cysts in intermediate hosts that can continue these cycles where those intermediate hosts are ingested.

Finally, another area that needs further study is to determine the mechanisms of how *Eimeria* overwinter in hibernating animals and the importance of these mechanisms to their maintenance in natural populations.

Ubiquitous, Neglected, and Complex: Untapped Biodiversity

The number of species of eimeriid coccidia is potentially staggering because these parasites have been found to infect all vertebrate and some invertebrate species that have been sampled for them. Unfortunately, most parasite surveys of vertebrates have concentrated only on their helminth and/or arthropod companions and largely have ignored their *Eimeria* (and other protozoan) parasites. For example, looking at the 5 classes of vertebrates we know only the following about their coccidia to date:

Amphibia (Frogs, Toads, and Salamanders)

This class has 3 orders, 56 families, 464 genera, and 6,009 species. Only 14 of 56 (25%) extant families, 28 of 464 (6%) genera, and 45 of 6,009 (< 1%) species have ever been examined for Coccidia. From these surveys 89 identifications were made. These include 52 coccidia species described and given binomials: 38 *Eimeria*, 11 *Isospora*, 2 *Goussia*, and 1 *Hyaloklossia* species. In addition, 37 additional names appeared that researchers believe are not valid including: 10 species inquirendae (which are species of doubtful identity), 22 incertae sedis (which have been placed in an uncertain taxonomic position), and 5 nomen nuda (which are nude names without formal descriptions) are entered into the literature (Duszynski et al., 2007).

Aves (Birds)

No definitive summary exists yet for *Eimeria* or other Coccidia genera from the 2 superorders and 29 orders of about 10,000 extant bird species, but it is known that there are many *Eimeria* and *Isospora* species already described (along with at least 6 other coccidian genera) and that some of these species, especially some eimerians from chickens and turkeys, can be exceptionally pathogenic to their hosts.

Mammals

There are about 5,416 mammal species organized into 1,229 genera in 53 families placed into 29 orders (Wilson and Reeder, 2005). It is noteworthy that 13 of 29 (45%) orders have been looked at in detail for their coccidia including:

Soricomorpha (Insectivores)

In the insectivores, 4 of 7 (57%) families, 19 of 66 (29%) genera, and 37 of 428 (9%) species have been examined for their coccidia. From these surveys, 120 coccidia species were described including: 48 *Eimeria*, 22 *Isospora*, 5 *Cyclospora*, and 45 species inquirendae including *Coccidium*, *Cyclospora*, *Eimeria*, *Gousseffia*, *Isospora*, and "Coccidia" species (Duszynski and Upton, 2000).

Primates (Monkeys)

Only 7 of 13 (54%) families, 14 of 60 (23%) genera, and 18 of 233 (8%) species have been examined for their coccidia. From these surveys 28 coccidia species were described including: 7 *Eimeria*, 8 *Isospora*, 1 *Cyclospora*, and 12 species inquirendae (Duszynski et al., 1999).

Scandentia (Tree Shrews)

Tree shrews all are placed in 1 family. Only 2 of 5 (40%) genera and 4 of 19 (21%) species in this family have been examined for coccidians. From these surveys only 4 *Eimeria* species have been described (Duszynski et al., 1999).

Chiroptera (Bats)

Only 6 of 17 (35%) families, 37 of 177 (21%) genera, and 86 of 925 (9%) species have been examined for their coccidia. From these surveys 39 coccidia species were described including 31 *Eimeria* and 8 species inquirendae (Duszynski, 2002).

Lagomorpha (Rabbits)

When compared to other mammalian orders, the Lagomorpha is not diverse and contains only 2 extant families, but even with such a tractable group not much is known about their coccidian parasites, except for just a few species. Although species in both extant families have been studied (a little), only 5 of 12 (42%) extant genera and 25 of 96 (26%) species have been examined. From these surveys, 87 coccidia species were described including 3 *Besnoitia*, 3 *Cryptosporidium*, 73 *Eimeria*, 2 *Isospora*, 5 *Sarcocystis*, and *Toxoplasma gondii*, and 33 species inquirendae (Duszynski and Couch, 2013).

Marsupialia (Marsupials)

In most earlier classifications of mammals (for example, Nowak, 1991) all marsupials were placed in a single order, but results from molecular and genetic research tools in the last 15 years have directed mammalogists to partition them into 7 orders within 2 superorders: Ameridelphia (Didelphiamorphia, Microbiotheria, Paucituberculata), the American marsupials, and the Australidelphia (Dasyuromorphia, Diprotodontia, Notoryctemorphia, Peramelemorphia) the Australian marsupials (Wilson and Reeder, 2005). Duszynski combines the parasite data for these 7 orders into the original

Marsupialia, within which there are 21 families, 92 genera, and 331 species. From all pertinent surveys 154 coccidia species are named including: 1 *Besnoitia*, 6 *Cryptosporidium*, 56 *Eimeria*, 1 *Isospora*, 11 *Klossiella,* 10 *Sarcocystis* species, *Toxoplasma gondii*, and 68 species inquirendae from 85 of 331 (26%) marsupial species examined. These species are found in 14 of 21 (67%) families examined for coccidian parasites, in 45 of 92 (49%) genera, and in only 85 of 331 (26%) marsupial species that have been examined for coccidian parasites (Duszynski, 2016).

Carnivora (Carnivores, Cats, and Dogs)

This order is separated into 2 suborders, Caniformia (9 families) and Feliformia (6 families), and these 15 families have 126 genera and 287 species (Wilson and Reeder, 2005). There are about 207 valid coccidian species named including 5 *Besnoitia*, 1 *Caryospora*, 10 *Cryptosporidium*, 1 *Cyclospora*, 53 *Cystoisospora*, 39 *Eimeria*, 3 *Hammondia*, 6 *Hepatozoon*, 9 *Isospora*, 1 *Neospora*, 78 *Sarcocystis*, and *Toxoplasma gondii*. There also are about 483 incompletely named species (species inquirendae) recorded from carnivores that fit taxonomically into these taxa as genus names only, or less. These species are found in 11 of 15 (73%) families, in 30 of 126 (24%) genera, and in only 48 of 287 (17%) carnivore species that have been examined for coccidian parasites.

Rodentia (Mice, Rats, Squirrels, etc.)

There are 33 families, 481 genera, and 2,277 extant species of rodents (Wilson and Reeder, 2005). Although there is no group-by-group summary to date, it is estimated that only about 15% of all rodent species have been surveyed for coccidia. From these surveys about 450 *Eimeria* and *Isospora* species have been described (Duszynski and Upton, 2001).

Remaining 15 Mammalian Orders

No definitive summaries exist to date although some coccidia species in 8 genera have already been described from a few of their species.

Pisces (Fish)

No definitive summary exists yet for the 32,500 extant fish species although many coccidian species in at least 6 genera already have been described (unpublished data).

Reptiles (Snakes)

Only 6 of 17 families (35%), 110 of 457 genera (24%), and 208 of 3,108 snake species (7%) have been examined for their coccidia. From these surveys, 302 coccidia species were described including 52 *Caryospora*, 2 *Cryptosporidium*, 4 *Cyclospora*, 66 *Eimeria*, 7 *Isospora*, 22 *Sarcocystis*, 1 *Wenyonella*, 2 *Tyzzeria*, and 148 species inquirendae, the latter including 3 additional genera (*Dorisiella, Globidium*, and *Pythonella*) (Duszynski and Upton, 2010).

Reptiles (Turtles)

The order Testudines is separated into 2 suborders, Cryptodira (11 families) and Pleurodira (3 families), and these 14 families have 96 genera and 351 species (Uetz, et al., 2018). Surprisingly, at least 1 species in 10 of the 14 (71%) families has been examined for coccidian parasites, but only 30 of 96 (31%) genera and 61 of 351 (17%) turtle species have been examined for coccidia and 100 coccidia species in 7 genera are known: 2 *Caryospora,* 66 *Eimeria,* 3 *Isospora,* 1 *Sarcocystis*, and 28 species inquirendae (4 *Coccidium,* 1 *Caryospora*, 9 *Cryptosporidium*, 5 *Eimeria,* 1 *Manotella*, and 8 *Sarcocystis* species [?]) (Duszynski and Morrow, 2014).

Reptiles (Alligators)

The order Crocodylia includes 27 species of alligators, caimans, crocodiles, and gharials. Duszynski et al. (2020) reported the blood and intestinal apicomplexans know to date from these reptiles and concluded that 17/27 (63%) had 16 apicomplexan species unique to them including: 8 Eimeria, 1 Haemogregarina, 4 Hepatozoon, 2 Isospora, and 1 Progarnia species; they also reported an additional 46 apicomplexan-like forms that were considered species inquirendae that await further study.

Reptiles (Lizards)

No definitive summary exists yet for all lizards, although many apicomplexans in 8 genera already have been described (unpublished data). This is now a work in progress.

There are approximately 62,150 extant vertebrate species known on Earth and, to date, there is comprehensive, systematic survey data on 16 vertebrate groups, (amphibians, 13 mammalian orders, snakes, turtles, crocodiles) which comprise 11,787 species, but only 634 (5.3%) of these species have been examined for coccidia and from them about 1,146 species have been named in the literature or about 1.8 coccidia species per host species examined. Given that some host species (for example, chickens, rabbits, and others) have 10 or more *Eimeria* species that may be unique to them, and that even domestic animals, whose parasites have been studied for decades, have had new *Eimeria* species described from them recently, it is clear that only a fraction of the number of *Eimeria* (and other coccidia) species that occur in vertebrates have been described to date. Using numbers that are now out of date, Levine (1973) estimated that more than 45,000 species of *Eimeria* would be found if all vertebrate species were examined. This is a gross underestimation, but it points to the urgent need for more work in this area, especially given the alarming rate of habitat destruction

and vertebrate species extinctions occurring worldwide. If we assume, conservatively, that every vertebrate species on Earth is host to (minimally) 2 coccidia species unique to it, we could expect to find at least 124,300 total coccidia species. The 1,800 or so coccidia species currently known is only 1.4% of the number of species that likely exist in Earth's vertebrates. In other words, 98.6% (or more) of the coccidian parasites of vertebrates are yet to be discovered! Clearly, there is lots of work to be done.

Ubiquitous, Neglected, and Complex: Specificity

Eimeria species demonstrate both site and host specificity, but to somewhat different degrees. The majority of species for which endogenous development is known undergo development within certain cells of the gastrointestinal tract, but not all species are found in this location. *Eimeria stiedai* undergoes development in epithelial cells of the bile duct and parenchymal cells of the liver of rabbits. Other species have been found to develop in cells of the gallbladder (goat), placenta (hippopotamus), epididymis (elk), uterus (impala), genitalia of both sexes (hamsters), bile duct (chamois), liver parenchyma (wallaby), and pyloric antrum (kangaroo) (Duszynski and Upton, 2001). *Hepatozoon* species develop in the blood of vertebrates and in arthropods, *Klossiella* species develop in kidney epithelial cells, and *Cystoisospora, Sarcocystis, Besnoitia,* and *Toxoplasma* have heteroxenous life histories. Once within their specific organ system of choice, *Eimeria* species seem to be limited to specific zones, specific cells within that zone, and specific locations within those cells. Thus, 1 species may be found only in the middle third of the small intestine and another only in the cells of the cecum. Within their specific region 1 species may be found only in cells at the base of the crypts of Lieberkühn, a second species in epithelial cells along the villi, and a third species in endothelial cells of the lacteals in the villi. Some species develop below the striated (microvillus) border of endothelial cells, but above the nucleus, others below the nucleus and a few within the nucleus. And a few other species or genera associate closely with the brush border of the epithelial cells and may even be extracytoplasmic (for example, *Cryptosporidium* species).

The degree of host specificity seems to vary from host group to host group; it's been studied best in mammals, and to a lesser degree in birds, especially domesticated stock or flock animals. *Eimeria* species from goats cannot be transmitted to sheep and vice versa (Lindsay and Todd, 1993), but *Eimeria* from cattle (*Bos*) are found to infect American bison (*Bison*). *Eimeria* species from certain rodents (Sciuridae) seem to cross host generic boundaries easily (Wilber et al., 1998), whereas other rodent species (Muridae) may cross species, but not genus boundaries (Hnida and Duszynski, 1999). In the Lagomorpha, 6 of 17 (35%) *Eimeria* species reported from cottontails (*Sylvilagus* spp.) are experimentally infective for the tame rabbit (*Oryctolagus cuniculus*). Similarly, some species from gallinaceous birds can be transmitted only to congeners, whereas others can be cross-transmitted between genera. One species has even been reported to cross familial lines, but this seems rare (De Vos, 1970). It also is known that *Eimeria separata* Becker and Hall, 1931, from rats will infect certain genetic strains of mice and that genetically altered or immunosuppressed mammals are susceptible to infection with *Eimeria* species to which they otherwise might be naturally refractory. Thus, numerous biotic interactions, particularly the genome of both parasite and host, must contribute to the host specificity, or lack thereof, attributed to each *Eimeria* species.

Ubiquitous, Neglected, and Complex: Significance in Biomedical Research

All members of the protozoan phylum Apicomplexa are obligate intracellular parasites. In addition to the *Eimeria*, many of their closely related cousins (for example, *Isospora*, *Sarcocystis*, *Tyzzeria*, and others) can cause economically important diseases in domesticated, and sometimes wild, animals. Other related forms in the phylum (for example, *Toxoplasma*, *Cyclospora*, *Cryptosporidium*, and *Plasmodium*) cause human disease in hundreds of millions of people worldwide. Classical genetic studies have been limited by the intracellular habitats of all these organisms and/or by the complex life cycles of some of them. However, development of pulsed-field electrophoresis, DNA sequencing, PCR, and related techniques has allowed good progress in understanding of the genomes of these parasites. In fact, the complete genome sequence of the most pathogenic human malaria parasite, *P. falciparum*, is now known (Gardner et al., 2002).

Recently, use of some of these molecular techniques has shown that a number of apicomplexan parasites (*Eimeria*, *Plasmodium*, and *Toxoplasma* species) have 2 extrachromosomal DNAs: 1) A small mitochondrial genome and 2) a unique 35 kb circular DNA. Sequencing and other molecular data suggest that the 35 kb DNA may be related to plastid DNA (plDNA). This plDNA should be of keen interest to researchers because its true origin, cellular location, function within these parasite cells, and relation to their nuclear genomes, are still a mystery. The exciting appeal for studying the plDNAs is their potential as specific targets for chemotherapeutics: Potential "silver bullets" to control the undesirable parasites of humans and their domesticated animals

that reside within the Apicomplexa. If the sequence of plDNA genes differs significantly from the genes for similar functions in their hosts, then such a function-mediated gene may prove an ideal target for development of chemotherapeutics that could be efficacious and nontoxic, while not inducing resistance. If plDNAs prove to be a common or universal feature of members of the Apicomplexa, they also have great potential for use as a phylogenetic yardstick to determine the evolutionary origin and history of apicomplexan parasites.

Ubiquitous, Neglected, and Complex: Human and Veterinary Medicine

Unlike members of the closely related genus *Cyclospora* and its more distantly related cousin *Cryptosporidium*, there is no evidence that any *Eimeria* species infect humans. In fact, both the nearest relatives to the primates, the Scandentia and the prosimians within the Primates are infected only by *Eimeria* species, whereas the anthropoid primates, which include the hominids, are only infected by *Cystoisospora* species, such as *Cystoisospora belli*, an important human pathogen.

Wild animals other than anthropoids (for example, mice, rabbits, and moles) almost always are infected with 1 or more *Eimeria* species at one or more times during their life and some might be infected during their entire lives with several species that cycle through them constantly. Given their ubiquitous nature, *Eimeria* species probably do not often cause discernable pathology or disease under natural conditions, but exceptions exist. For example, *E. bovis* (Züblin, 1908) Fiebiger, 1912 in cattle, *E. tenella* (Raillet and Lucet, 1891) Fantham, 1909 in chickens, and *E. stiedai* (Lindemann, 1865) Kisskalt and Hartmann, 1907 in rabbits all are known to be highly pathogenic in their respective hosts and, recently, another pathogenic species, *E. brachylagia* Duszynski et al., 2005, was found to cause heavy intestinal infections, some of which resulted in deaths in the endangered Columbia Basin pygmy rabbit, *Brachylagus idahoensis*, in Washington and Oregon, United States (Duszynski et al., 2005). *Eimeria chinchillae* has a broad host range and is also highly pathogenic, causing bloody diarrhea, anorexia, severe lesions in the intestines, and ultimately leads to the death of infected animals. It may also cause neurological symptoms (De Vos and Westhuizen, 1968; De Vos, 1970).

When animals are concentrated together, enhancing transmission of *Eimeria* via its rapid, direct life cycle, some species will cause a disease condition, coccidiosis. Coccidiosis is recognized as a major health hazard: During intensive husbandry of domestic animals; in wild, captive animals such as those in breeding and research facilities and zoos; in wild animal populations when habitat is lost and crowding occurs; and in wild animal species that have great reproductive potential and are protected by laws so that their populations increase inordinately (for example, kangaroos in Australia). All of these conditions are the result of human intervention or perturbation.

Coccidiosis is a serious problem in the poultry industry. In the United States alone, more than 4 *trillion* birds are raised annually and the United States Department of Agriculture estimated that loss to poultry farmers in the mid-1980s exceeded US$ 80 million when deaths, medicated feeds, and all added labor costs were considered. Worldwide expenditures, just for coccidiostats added to broiler feed, are estimated to be US$ 250–300 million annually. Once a flock becomes infected, especially with 1 or several of the more pathogenic species, a large percentage of the flock can die rapidly. Birds not killed outright by their infection become listless and are more susceptible to predators and other diseases. Even if they survive their infection, they have reduced feed efficiencies. In addition, *Eimeria* species are becoming widely resistant to the coccidiostats in feed. Similar morbidity and/or mortality and related events occur in cattle feedlots or wherever meat animals are congregated in large numbers.

Coccidiosis is also well documented in some wild species. *Eimeria gruis* Yakimoff and Matschoulsky, 1935 and *E. reichenowi* Yakimoff and Matschoulsky, 1935, for example, are common parasites of both whooping cranes and Sandhill cranes in North America and have been reported in other crane species in captivity. These species are considered an important cause of mortality in captive cranes and, during migrations when large numbers of several species of cranes congregate for lengthy periods at watering holes, can be responsible for illness and death in wild populations. Although the disease is generally limited to the intestinal tract in most animals, *Eimeria* infection in cranes may result in disseminated visceral coccidiosis, where endogenous stages from the gastrointestinal tract become disseminated throughout the body, via the blood or lymphatic systems. Nodules with meront and gamont stages are found in many organs, including lungs, air sacs, trachea, and nares. This disseminated visceral coccidiosis has caused the death of a number of captive Sandhill cranes and whooping cranes. Of the few *Eimeria* species known to have extraintestinal developmental stages, only the species in cranes can complete their life cycle in both the digestive and respiratory tracts (Carpenter, 1993).

Habitat Destruction, Coccidian Transmission, and Disease

Finally, as the human population continues to grow and agricultural development accelerates to try keep pace, natural places and their endemic faunas will decrease dramatically. The immediate effect of shrinking ecosystems (for example,

tropical rain forests, coastal estuaries/wetlands, temperate old-growth forests) is to concentrate both species and individuals into restricted, fragmented areas promoting increased transmission and exchange of parasites, especially those with direct life cycles with resistant oocysts, like many coccidians. Such close contact between host species and their parasites could allow these organisms to become agents of extinction as the host range(s) contract.

Fragmentation increases the edge-effect and can bring an influx of new host species into disturbed or agricultural habitats between fragments, introducing new coccidians and possibly leading to the development of new and more pathogenic strains. Changes in parasite species, intensities, or pathogenicity can have repercussions on the whole food web. The potential, either for domestic animals to become infected by coccidian parasites maintained in wild reservoir host populations, or the reverse, is a strong possibility. For example, we know that deer, elk, or bison can serve as reservoirs for *Eimeria* and other parasites for domestic livestock and wild rabbits can serve as reservoirs of *Eimeria* species capable of infecting domesticated rabbits. As humans breed themselves to the brink of extinction and habitat disappears globally at an ever-alarming rate, the potential for biological disaster from the exchange of ubiquitous protozoan parasites, like *Eimeria* species and its close relatives, may destabilize food webs. Environmental stressors (for example, PCBs), which may compromise host immune systems, global climate change, which challenges the adaptability of host organisms, and the invasion of new parasites, from edge-dwelling hosts, all increase the potential for many apicomplexan parasites to become pathogenic; thus, the importance of disease should be expected to increase in shrinking ecosystems as a consequence of habitat destruction.

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10

Protozoa

Apicomplexa

Haemosporida (Order): The "Malaria Parasites"

Susan L. Perkins and Spencer C. Galen

Phylum Myzozoa

Subphylum Apicomplexa

Order Haemosporida

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Chapter 10

Haemosporida (Order): The "Malaria Parasites"

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Introduction

The term malaria refers specifically to a disease of humans that is caused by an infection of red blood cells (erythrocytes) and other cells by protozoan parasites in the genus *Plasmodium* and transmitted by mosquitoes in the genus *Anopheles*. The pathology of this disease results from 3 primary sources: 1) Episodic fevers that are caused by the cyclic rupturing of host erythrocytes; 2) anemia that follows from infection of host erythrocytes and their subsequent death; and 3) clogged capillaries from the combination of a loss of elasticity of infected erythrocytes as well as parasites triggering host erythrocytes to express surface proteins that make them likely to cling to one another. Nearly half a million people still die from malaria worldwide every year (Cibulskis et al., 2016; WHO, 2021), primarily in sub-Saharan Africa and other tropical regions. No vaccine is yet available on a global scale, but an RTS,S vaccine against the most pathogenic species (*Plasmodium falciparum*) has recently been approved and is being deployed in Ghana (WHO, 2023). Several drugs have been used as prophylaxis or treatment, but the parasites have evolved resistance to these compounds in many regions of the world. The human parasites are only a tiny fraction of the very diverse clade Haemosporida (sometimes called Haemospororida) which contains over 600 described species of these protozoan parasites occurring in many different species of reptiles, birds, and other mammals. All haemosporidians use a vertebrate host and a biting dipteran (fly) vector during different stages of their life cycles. Non-human haemosporidians are sometimes colloquially referred to as the malaria parasites, due to their close relationships and similar life cycle to the parasites that cause this disease in humans.

History of Knowledge of Malaria

Symptoms of malaria, particularly its regularly spaced fevers, have been described by writers in antiquity going back as far as 5,000 BCE in China and more than 3,000 years ago in India, Sumeria, and Egypt. The writings of ancient Greece describe characteristic symptoms of *Plasmodium falciparum*, *P. vivax*, and *P. malariae* and Alexander the Great is believed to have died from *P. falciparum* while attempting to travel to India in 323 BCE (Carter and Mendis, 2002). It is thought that both European colonists and the enslaved West Africans that they transported to the New World brought malaria parasites, and by the mid-19th century, malaria was a common and endemic disease throughout the tropical and temperate regions of North America and South America (Carter and Mendis, 2002).

Despite the ubiquity and severity of malaria, the root cause of this disease remained largely a mystery, with only the link between smelly, swampy regions and the resulting symptoms as a clue (the word malaria stems from the Italian words for bad air, namely, **mal** + **aria**). Eventually, in the late 1800s a series of scientists, most notably Charles Laveran, began to piece together that tiny specks in the blood of sick humans, later known to be the blood stages of the parasites, were associated with the characteristic fevers of the disease. How it could pass from one human to another was not known until 1897, when Ronald Ross, a British Army doctor, showed that there were cells that could be found in the saliva of *Anopheles* mosquitoes that had fed on birds that were somewhat similar to those that he observed in the blood of sick human patients. Ross was awarded the Nobel Prize in Physiology or Medicine in 1902 for this major piece of the discovery, but the other aspects of the malaria life cycle remained unknown for many decades to come, namely, where the sporozoites went between the time they were injected into the host by the mosquito and when they appeared in the bloodstream of the same host.

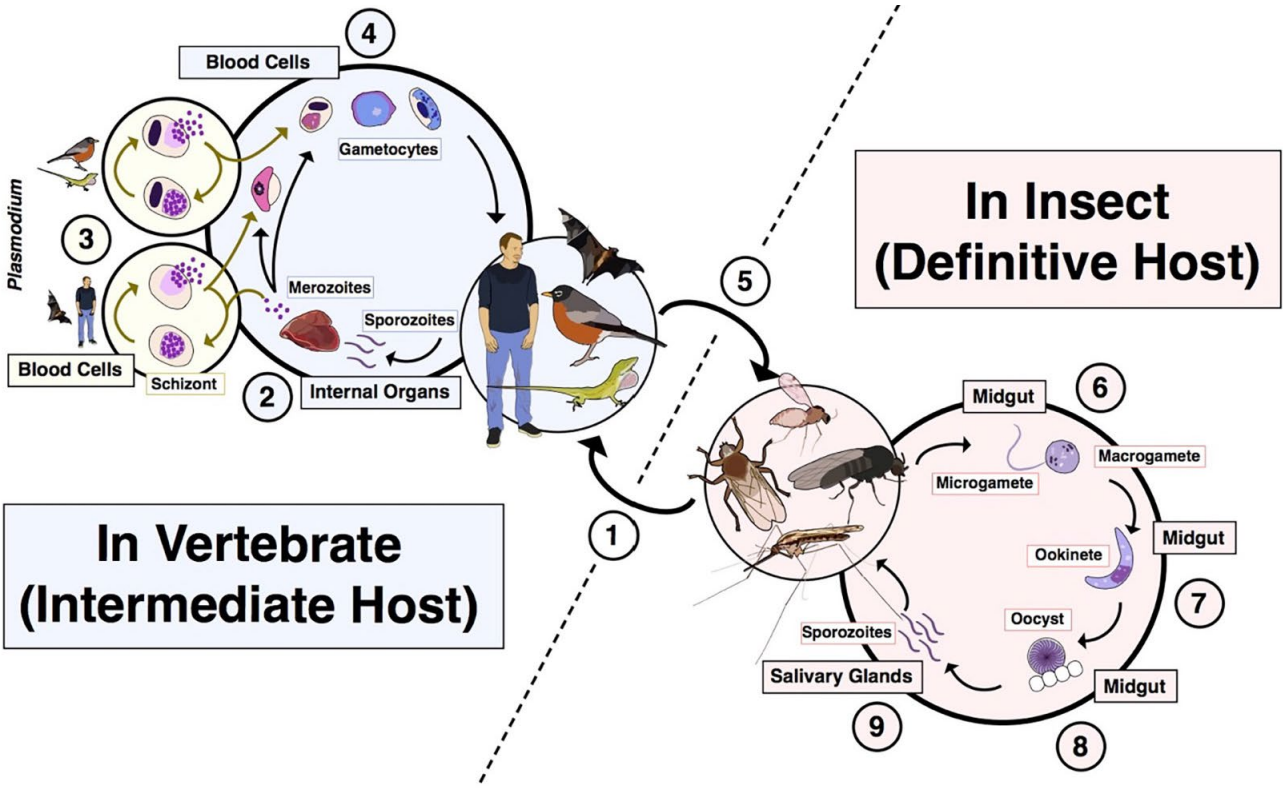

Figure 1. Generalized life cycle for haemosporidian (malaria) parasites. 1) An infected dipteran takes a blood meal from a vertebrate and sporozoite stages are injected into the bloodstream along with its saliva. 2) The sporozoites travel through the blood until they reach specific cells in internal organs where they will undergo rounds of asexual division. In human malaria parasites and many others of mammals, this occurs in cells of the liver. 3) For *Plasmodium* parasites, there are additional rounds of asexual division in blood cells as well. This can occur in both anucleated red blood cells of mammals (bottom) and in nucleated blood cells of birds and squamates (top). 4) Eventually, a developmental switch triggers the development of gametocytes, or the transmission stages. These stages are either of future male function (microgametocytes) or female function (macrogametocytes). These stages are highly variable across the diversity of haemosporidians. The illustration depicts gametocytes of *Plasmodium falciparum* (lower left), a lizard *Plasmodium* (second from left), a *Leucocytozoon* (third from left), and a *Haemoproteus* (fourth from left). 5) If a dipteran vector feeds from an infected vertebrate host, she will pick up gametocytes as part of that blood meal. 6). Once inside the fly's midgut, the gametes undergo exflagellation, forming the macrogamete (female) and multiple microgametes (male). 7) After fertilization, the zygote transforms into a motile stage known as the ookinete, which penetrates the wall of the fly's midgut. 8) A cyst, known as the oocyst forms and meiosis occurs at this point. 9) Sporozoites rupture from the oocyst and travel to the fly's head, coming to reside in the salivary glands to await the next blood meal. Source: S. C. Galen, 2019. License: CC BY-NC-SA 4.0.

Malaria Today

Today, malaria is still one of the largest public health burdens in the world (Sachs and Malaney, 2002; WHO, 2021; 2023) with as many as a quarter of a billion new cases arising each year. The number of cases and particularly the number of deaths has fallen recently due to improved prevention measures such as insecticide-infused bed nets, better diagnostics, and improved treatments, however, there is still plenty of reason to be cautious. The drug artemisinin (and its derivatives), which is the first line of treatment in all malaria endemic-countries, is now at risk of lower efficacy as resistance has evolved in several endemic regions and insecticide resistance remains a looming and potential problem.

General Life Cycle

All members of the order Haemosporida follow the same generalized life cycle, which is obligately heteroxenous (multiple hosts), alternating between a vertebrate and a biting fly such as a mosquito, midge, louse fly, sand fly, or black fly (Figure 1). Take as an example the bite of an already infected insect (Figure 1, point 1). Several groups of flies use blood as a source of proteins and lipids with which to make their eggs. If a fly is infected with haemosporidians, when she feeds from the vertebrate, the stages of the parasite known as sporozoites will be injected into the vertebrate bloodstream along with her saliva. Sporozoites will travel through the bloodstream until they come to the liver or other

target tissues, where they will invade the host cell and begin to asexually divide (Figure 1, point 2), often making thousands of daughter cells within just a few days. In some groups of haemosporidians, namely the genus *Plasmodium*, after this first round of tissue schizogony some of the parasites will leave the tissue stages and invade blood cells (typically red blood cells; Figure 1, point 3), where they transition into the asexual feeding stages known as trophozoites. During this point in the life cycle, the parasites digest the hemoglobin present in the hosts' red blood cells, eventually forming a crystalline compound known as hemozoin. These cells will then undergo additional asexual division and burst from the host cell, going on to infect new blood cells. However, the majority of the genera in the order Haemosporida do not asexually replicate in the host's blood cells, but instead continue their cycle in other tissues such as the epithelium of the lungs or spleen. All members of the order will at some point show the transmission stages, known as gametocytes, in the host blood cells (Figure 1, point 4). These cells exist as different sexes; females are known as macrogametocytes and males are known as microgametocytes. When another biting fly comes to feed on this host, the gametocytes will be taken up as part of her blood meal (Figure 1, point 5). Within the midgut of the fly, the gametocytes undergo a process called exflagellation, as the blood cells themselves begin to be digested. The microgametocytes split into several smaller microgametes, each of which can fertilize an exposed macrogamete (Figure 1, point 6). Once fused, the parasite exists as a motile stage called an ookinete (Figure 1, point 7). These cells push through the cells of the insect's gut and encyst on the outer edge, in a structure called the oocyst (Figure 1, point 8). Thousands of sporozoites emerge from each oocyst and migrate to the insect's salivary glands, where they wait until her next blood meal to infect a new vertebrate host (Figure 1, point 9).

Because the sexual component of the malaria parasite's life cycle occurs within the insect, technically it is the insect that should be referred to as the definitive host, with the human or other vertebrate host referred to as the intermediate host. The dipteran insect is, however, often referred to as the vector of the malaria parasite as it does transmit the parasite between humans or other vertebrate hosts. The complete life cycle has been worked out in detail for only a few of the species of haemosporidians that infect non-human hosts, with most assumed to follow the same stages as their close relatives in the same genus. Although the use of different insect host groups (Table 1) is known for some of these parasites, the vast majority of haemosporidians are transmitted by unknown species of flies.

Diversity of and Relationships within the Family Haemosporida

The species of *Plasmodium* that commonly infect humans are the best known and most intensively studied haemosporidians, though they are of very minor importance in terms of the overall diversity of these parasites. Birds or their dinosaur precursors are most likely the original vertebrate hosts of the malaria parasites and still harbor the greatest diversity of the haemosporidians, both in terms of the number of described species that use birds as hosts, but also their geographic range (Valkiūnas, 2004). Numerous other vertebrate groups are hosts to species of haemosporidians, including monkeys, bats, lizards, and turtles. Haemosporidians are typically not capable of infecting hosts outside of their major host group, that is, bird malaria parasites cannot infect humans and vice versa. However, within their major host groups some malaria parasites (especially the avian malaria parasites) can infect many different species. For example, the cosmopolitan avian malaria parasite *P. relictum* has been recorded to infect over 300 different species of birds throughout the world (Valkiūnas et al., 2018).

Table 1. Major groups of haemosporidians, with vertebrate and dipteran hosts used.

Genus	Vertebrate hosts used	Dipteran insect hosts used
Plasmodium	Primates, rodents, bats, birds,* squamates,* ungulates*	Anopheline and culicine mosquitoes
Hepatocystis	Primates, bats	Culicoides midges, others?
Polychromophilus	Bats	Nycterbid flies
Nycteria	Bats	Unknown
Haemoproteus	Birds	Hippoboscid flies
Parahaemoproteus	Birds	Culicoides midges
Haemocystidium	Squamates, turtles	Tabanid flies, others?
Leucocytozoon	Birds	Simulids (black flies)

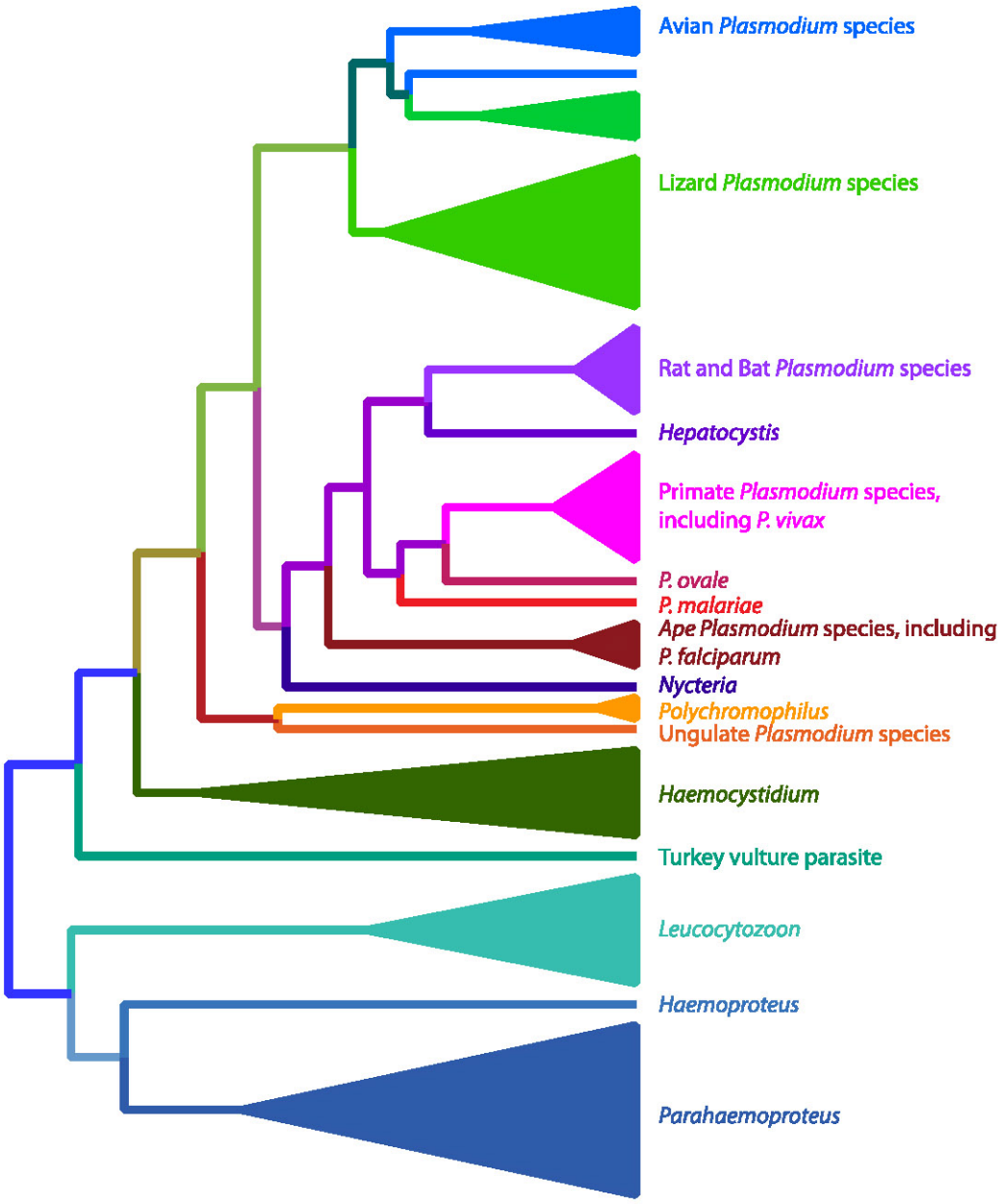

Figure 2. Phylogenetic relationships of the haemosporidian (malaria) parasites. An analysis of more than 20 genes (Galen et al., 2018) resulted in this hypothesis of the evolutionary relationships of various clades of haemosporidians. This tree supports that birds were the original hosts of these parasites with either a single introduction into mammalian hosts and a subsequent reinfection of birds and lizards by *Plasmodium* parasites, or that there were 2 invasions into mammals. This tree would make the genus *Plasmodium* polyphyletic as not all members share a common ancestor, however this would mean that significant taxonomic changes need to occur. Source: Galen et al., 2018. License: CC BY-NC-SA 4.0.

Classification

The classification of the malaria parasites has been extremely fluid throughout history. For the bulk of this time, taxonomic groups including genera and families were primarily structured around 2 primary characteristics of the parasite species: 1) If the parasite reproduced asexually in the host blood (known as erythrocytic schizogony) and 2) whether or not hemozoin pigment was visible in the blood stages of the parasite. These traits were considered to be the most important in the separation of the parasites into genera and served as the basis for the creation of 4 families within the order (Levine, 1988).

The advent of using DNA sequences as characters with which to understand the evolutionary history and

relationships among organisms drastically changed the hypotheses of the relationships within Haemosporida. The first molecular systematic study of these parasites used the 18S ribosomal small-subunit gene and a small set of taxa including the important human parasites, *Plasmodium falciparum* and *P. vivax*, as well as parasites found in rodents and 2 species from birds (Waters et al., 1991). The resulting topology showed a close relationship between *P. falciparum* and *P. gallinaceum*, a parasite that infects chickens, and the authors naturally concluded that humans had acquired the virulent *P. falciparum* following a host switch after chickens were domesticated. However, subsequent studies, particularly those using other genes, did not support the human/chicken connection (Ayala et al., 1999; Perkins and Schall, 2002) and instead showed that the human- and bird-infecting *Plasmodium* lineages are distantly related. These later studies also established that the avian parasites in the genus *Leucocytozoon* were likely an early-diverging lineage (Perkins and Schall, 2002). Recently, multiple genes from a large number of different haemosporidian parasites were sequenced and used to create the most comprehensive phylogeny to date (Figure 2) (Galen et al., 2018). These results highlighted the complex evolution of the Haemosporida and show that the original characters used to define clades have likely evolved more than once. This updated phylogeny also showed that the taxonomy of this group of parasites needed to be revised. For instance, most recent analyses have recovered parasites that have been classified as the distinct genus *Hepatocystis* as closely related to the human-infecting *Plasmodium* species (Perkins and Schall, 2002; Galen et al., 2018). Conversely, many parasites classified as *Plasmodium* because they show schizogony in blood cells and clear hemozoin pigment, have been shown not to be part of a monophyletic group that contains the other *Plasmodium* species, including the type species of the genus.

Malaria Parasites of Birds

Malaria parasites are practically ubiquitous in birds with a cosmpolitan distribution. Several hundred species have been described from the genera *Plasmodium*, *Haemoproteus*, *Parahaemoproteus*, and *Leucocytozoon* (Table 1; Figure 2), making the bird-infecting malaria parasites the most species rich group within the Haemosporida.

Avian malaria has been instrumental in studies of the disease ever since it was first discovered in the late 1800s. Ronald Ross (Figure 3), who won the Nobel Prize for his discovery that mosquitoes were responsible for transmitting the parasites from person to person, first did experiments on birds infected with *Plasmodium* parasites (Rivero and Gandon, 2018). Bird systems were also what allowed the discovery that the parasite first completes 1 or more exoerythrocytic stages before it begins to infect the blood cells of the host (Huff and Coulston, 1946). Also, experiments using avian malaria were useful for understanding immunity to *Plasmodium* infection by testing the efficacy of early anti-malarial drugs (Tonkin and Hawking, 1947; Rivero and Gandon, 2018). The method of inoculating naive hosts with sporozoite stages that had been rendered inactive, one that is being tested in humans now (2019), was first developed in an avian malaria system (Rivero and Gandon, 2018). A large number of researchers continue to use avian malaria as a model system for studying parasite-host interactions and diversification. The attractiveness of avian malaria as a system lies in the fact that it is relatively easy and cost-effective to sample large numbers of birds from a variety of species in a given habitat via mist-netting and drawing a small blood sample. Haemosporidian-specific primers are available that allow the samples to be rapidly screened for the presence of parasites and identified to lineages by sequencing. Comprehensive and publicly accessible databases can then be assembled (Bensch et al., 2009) so that comparative studies of host use and diversification are possible. Through this type of molecular work on avian systems, over 3,000 different lineages of malaria parasites have been reported from all over the world, with some authors estimating that the number of species of avian malaria parasites may be as high as 10,000 (Bensch et al., 2004). One pattern that has emerged from this work is that avian malaria parasites can exhibit host generalism (with a broad host range), infecting large numbers of distantly related species of birds, or host specialization (with a narrow host range), infecting a small number of closely related host species (Martínez-de la Puente et al., 2011; Svensson-Coelho et al., 2014; Ellis et al., 2015). The reasons for the higher abundance, diversity, and variation in host infection patterns exhibited by the avian malaria parasites relative to other malaria parasites are poorly understood and have led to an increased interest in avian haemosporidian research in recent years (Bensch et al., 2009).

Although important in early laboratory studies of malaria, the popularity of using birds as a model system waned substantially when the rodent malaria parasites were successfully cycled in laboratory mice. However, because the rodent malaria system involves just a small set of closely related parasite species that are used to infect an unnatural host species, there has been a recent resurgence in using birds as experimental systems with which to study the biology of malaria parasites (Rivero and Gandon, 2018). These studies have been accelerated by the ability to sequence the first genomes of avian malaria parasites (Bensch et al., 2016; Lutz et al., 2016a; Böhme et al., 2018) as well as transcriptome studies

Figure 3. Ronald Ross. Sir Ronald Ross (1857–1932) was a British medical doctor whose work in India on both avian and human malaria parasites resulted in the discovery that mosquitoes transmit infective stages between vertebrates. He won the Nobel Prize in Medicine in 1902. Photo source: United States National Library of Medicine Digital Collections, https://collections.nlm.nih.gov/catalog/nlm:nlmuid-101427700-img. Public domain.

that can be done quite easily in bird hosts (Videvall et al., 2015; Weinberg et al., 2018).

Examples of some of the questions that are easily addressed using avian malaria parasites include studies on parasite virulence in relation to parasitemia in the host (Palinauskas et al., 2018) and costs to the reproduction and survival of parasites in the mosquito vector (Pigeault et al., 2015; Yan et al., 2018; Vézilier et al., 2012). One of the most exciting recent discoveries involving avian malaria parasites was that infected birds showed a marked shortening in their telomeres, the ends of chromosomes, which are thought to be related to life span in vertebrates in general (Asghar et al., 2016; Remot et al., 2022). This discovery prompted similar examination of telomere length in malaria-infected humans and showed that cell-death is induced by *Plasmodium* infection in our species as well (Asghar et al., 2017).

Avian malaria parasites have also been shown to have negative impacts on naïve host populations in at least one tragic case where the parasite was accidentally introduced to a region. In the early 1800s, *Culex* mosquitoes were accidentally introduced to the Hawaiian Islands and a few decades later, *Plasmodium relictum* was also brought there. An endemic transmission cycle was established, which quickly spilled into the native Hawaiian avifauna and likely contributed to their extinctions of some species (van Riper et al., 1986; Atkinson and Samuel, 2010; Samuel, et al., 2011).

Malaria Parasites of Squamate Reptiles (Class Reptilia: Order Squamata)

As with birds, the malaria parasites of reptiles are also geographically widespread (occurring on every continent except for Antarctica) and diverse, with over 100 described species (Telford, 2008). They infect a large number of squamates as hosts including over 10 different families of lizards and 3 species in snakes.

In lizards, the pathology of haemosporidians has only been well studied in 2 different systems, with varying results. In western fence lizards (*Sceloporus occidentalis*) that are infected with *Plasmodium mexicanum*, serious fitness consequences from infection were observed (Schall, 1990). Male lizards with these parasites were less likely to be able to defend a territory and infected female lizards laid fewer eggs per clutch. However, in another system, the Saban anole and its *Plasmodium* parasites in the Caribbean, these results were not found—infected and uninfected lizards showed similar reproductive success and survival (Schall and Staats, 2002).

The malaria parasites of lizards have been used as a model system to study a variety of host-parasite relationships, including the role of these parasites on sexual selection (Schall, 1983; Schall and Staats, 1997), the evolution of sex ratios for optimal transmission (Schall, 2000; 2009; Osgood and Schall, 2004; Neal and Schall, 2010; 2014; Neal, 2011), and island biogeography and parasite diversification (Mahrt, 1987; Perkins, 2001; Falk et al., 2015).

Malaria Parasites of Rodents

The rodent malaria parasites represent an unusual case where the parasite was first discovered in the insect host as opposed to the vertebrate one. In the 1940s entomological surveys in what is now the Democratic Republic of the Congo discovered a new species of *Anopheles* mosquito and some of them were found to contain sporozoites in their salivary glands. Given that this was long before DNA sequencing

could be used to identify the hosts that they had fed on, assays that tested interactions with blood proteins were used, and rodents were identified as the likely source. A few years later, *Grammomys surdaster* (order Rodentia: family Muridae), the African woodland thicket rat were found infected with the parasites and the *Plasmodium* was successfully inoculated into white laboratory mice—and the rodent-malaria model system was born (Killick-Kendrick and Peters, 1978).

In a short span of time, 4 main species of rodent malaria were described and established as culture systems in laboratory mice. This model system played major roles in the early laboratory studies and characterization of malaria parasites, including early cell biology as well as genetic and immunological studies. The system was so important that the genome of a rodent malaria parasite, *Plasmodium yoelii*, was the very next to be sequenced following the publication of the *P. falciparum* genome (Carlton et al., 2002).

Malaria Parasites of Other Mammals

There are several other groups of mammals that are natural hosts for malaria parasites, including other primates, bats, and ungulates. However, generally these malaria parasites of other mammal groups have been less intensively studied than the model malaria parasites of humans and rodents.

Bats played an important role in the discovery of malaria parasites as it was Dionisi who first observed the cells in their blood as far back as 1898 (Perkins and Schaer, 2016). Given the dispersed nature of bats as hosts in the phylogeny of haemosporidians (Figure 2), they are also likely to be important transition hosts between bird hosts and other mammal hosts (Lutz et al., 2016b; Perkins and Schaer, 2016). Four primary genera have been found in various groups of bats worldwide. These include *Plasmodium* in Africa, *Hepatocystis* in Africa and Asia, *Nycteria* in Africa, and *Polychromophilus* from Africa, Europe, Central America, and South America. Several other monotypic genera (that is, a genus with a single species) have also been described from bat hosts, but their status will remain uncertain until genetic data can be collected. What was most interesting about the first molecular systematic studies of bat malaria is that they showed a very close relationship with the rodent malaria parasites that are so popular now as laboratory models (Schaer et al., 2013). The bat hosts of these parasites roost in trees that likely overlap ecologically with the arboreal thicket rats that serve as the natural hosts for the rodent-infecting *Plasmodium* species.

Several species of *Plasmodium* have been described from various ungulates including buffalo, goats, and small antelope. *Plasmodium* was also identified in a single white-tailed deer that had had its spleen removed in the southern United States (Garnham and Kuttler, 1980). Although deer are abundant in the eastern United States, the parasites in deer were not observed again until just recently, when several animals in Washington, DC, and other sites were shown to be infected by this parasite, now named *P. odocoilei* (Martinsen et al., 2016). Around the same time, other researchers also reported malaria parasites in hooved hosts ranging from goats in Africa to water buffalo in Thailand (Templeton et al., 2016). Phylogenetic analyses show that all ungulate malaria parasites discovered thus far are part of the same clade, but also that this clade is not truly part of the genus *Plasmodium*, but rather likely a distinct genus (Galen et al., 2018). The pathology of the white-tailed deer parasites and their tendency to infect and be virulent in very young animals is of interest to biomedical researchers, however, as although they are distantly related, this life history may mean that these parasites could serve as a model for *P. falciparum* infection in humans (Guggisberg et al., 2018; Perkins, 2018).

Malaria parasites have also been reported in 2 other groups of mammals: The colugos of Africa and elephant shrews of Malaysia (Perkins and Schaer, 2016). In both of these cases, there are many other species of haemosporidians known from the region, however, suggesting expanded host range. Nonetheless, the distribution of malaria parasites in mammals worldwide presents a puzzling pattern. There are no known haemosporidians from major groups of mammals such as carnivores or lagomorphs, and relatively few malaria parasite species have been described from the 2 largest orders of mammals (rodents and bats) and those that are known from these mammals have restricted geographic distributions.

Malaria Parasites of Humans

There are several species of *Plasmodium* that use humans as their hosts. In the past decade, many closely related lineages of parasites have been discovered to infect wild apes or other primates with a potential to also infect humans. The genetic divergences and host specificity among these novel ape malaria parasites are subjects of much study, thus it is likely imprudent to give an exact number of the taxa that do or could infect humans (McFadden, 2019). The 5 most common species that are found in humans are discussed below.

Plasmodium falciparum

Plasmodium falciparum, sometimes referred to as malignant tertian malaria, is the most virulent of the human-infecting species (the term tertian stems from the fact that the fevers from this infection become synchronized to every 2 days, and the Romans who first classified it as such did not use the concept of zero). It is widely distributed throughout

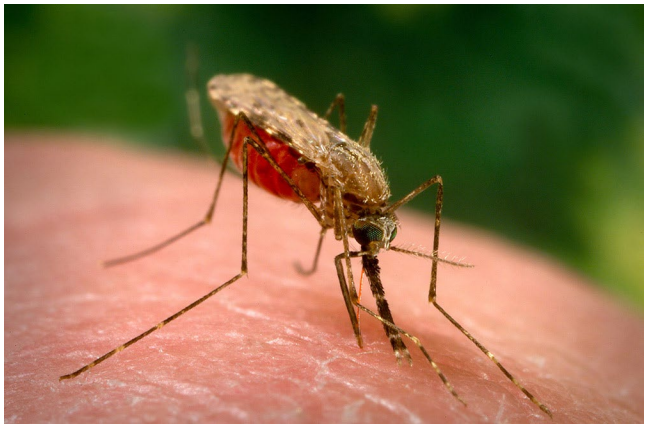

Figure 4. *Anopheles arabiensis*, one of the primary insect hosts of *Plasmodium falciparum*. Source: United States Centers for Disease Control and Prevention Public Health Image Library, image 18749; J. Gathany, 2014. Public domain.

the tropics, but is primarily concentrated in sub-Saharan Africa, Southeast Asia, and Oceania with known foci in South America. The reasons for the high virulence of this species are 2-fold. First, *P. falciparum* will invade any type of red blood cells and so can reach higher parasitemia than the other human parasites. Second, cells infected with *P. falciparum* become what might be thought of as sticky due to proteins expressed onto their surface as well as rigid and unable to bend, making it highly likely that they accumulate in capillaries causing a phenomenon known as sequestration. Sequestration in the brain or other vital tissues can cause death.

Plasmodium falciparum is transmitted among people primarily by the mosquito *Anopheles gambiae*, though many other species of *Anopheles*, such as *A. arabiensis* (Figure 4; see also Figure 5), are capable of transmission, depending on the geographic region (Molina-Cruz et al., 2016). In infected people the early trophozoite stages, which are called ring stages, are sometimes observed on thin blood smears but the mature stages are typically not observed due to the tendency of this species to sequester. *Plasmodium falciparum* is unusual amongst most mammal-infecting haemosporidians in that its gametocytes are crescent-shaped, rather than rounded (Figure 6A).

Virtually all human deaths attributed to malaria are caused by *Plasmodium falciparum*. It is currently present on all continents except for Europe (and Antarctica), but the largest proportion of fatalities is in children under 5 years-old who are living in sub-Saharan Africa. Because of its enormous global health importance, *P. falciparum* was the first malaria parasite—and one of the first organisms—to have its genome completely sequenced (Gardner et al., 2002).

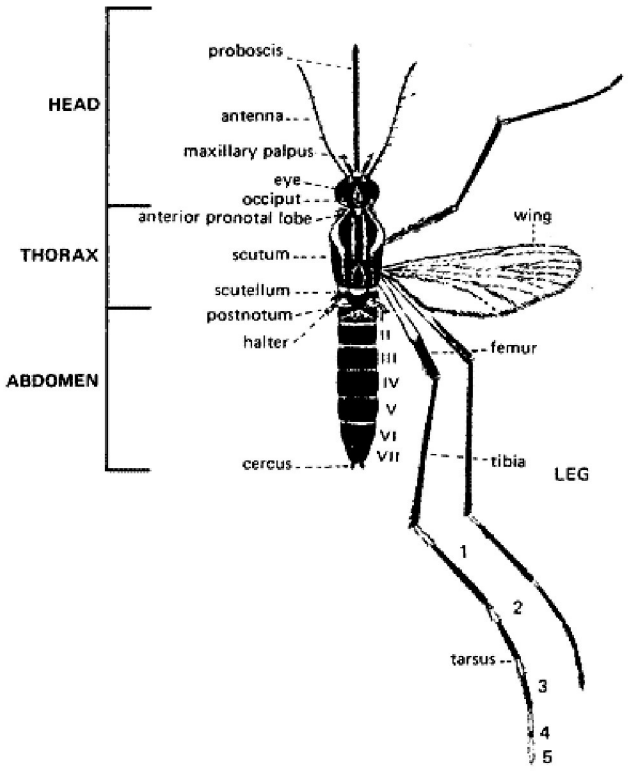

Figure 5. Mosquito morphology (female). Source: United States Centers for Disease Control and Prevention. Public domain.

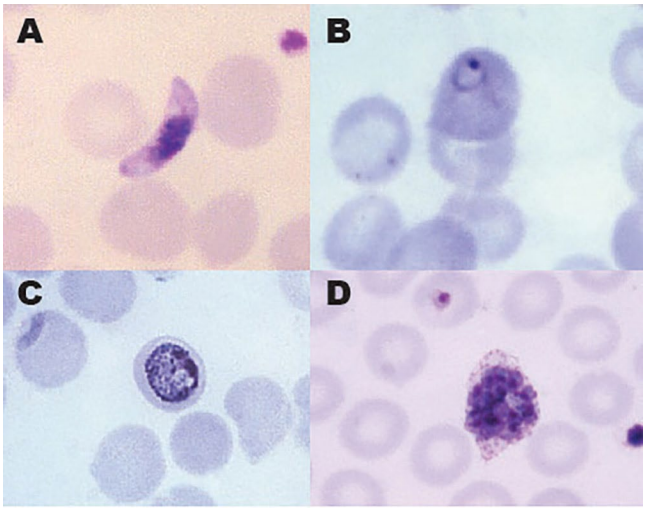

Figure 6. Stages of the 4 most common human malaria parasites. A) Gametocyte of *Plasmodium falciparum*. B) Ring stage of *P. vivax*. C) Trophozoite of *P. malariae*. D) Schizont of *P. ovale*. Source of photos: United States Centers for Disease Control and Prevention Public Health Image Library (A, image 4905; M. Melvin, 1966; C, image 5838; S. Glenn, 1979; D, image 5846; S. Glenn, 1979). Public domain.

Plasmodium vivax

Plasmodium vivax, also known as benign tertian malaria, is also globally distributed and in the not-so-distant past, was even present in eastern cities of the United States such as Washington, DC, Philadelphia, and New York City. Because it is so geographically widespread, the economic burden of this parasite is very large—almost 3 billion people worldwide live in areas where *P. vivax* is present (Battle et al., 2012).

Although *Plasmodium vivax* is colloquially referred to as benign, it also has major health effects on its hosts, similar to those of *P. falciparum*, including anemia, jaundice, and even cerebral malaria (Bourgard et al., 2018). However, from an evolutionary standpoint *P. vivax* is more closely related to what are typically referred to as the macaque malarias, including *P. cynomolgi* and *P. knowlesi* (Galen et al., 2018). Unlike *P. falciparum* which is flexible in the blood cells it infects, *P. vivax* has a strong preference for using the reticulocytes of the host (Galinski and Barnwell, 1996; Galen et al., 2018) and so has a much lower parasitemia with only ring stages typically present in circulating blood (Figure 6B). It also produces the gametocyte stages much earlier in the vertebrate host, as early as 4 days even before clinical symptoms might present, a factor that could promote its transmission to new mosquitoes (Bourgard et al., 2018). What is perhaps most notable about *P. vivax*'s life cycle, however, is its presence of stages that remain viable in the liver called hypnozoites (Markus, 1980) that can later trigger a relapse in the disease.

Unlike *Plasmodium falciparum*, it has not been possible to culture *P. vivax* parasites in vitro in the laboratory, therefore it has been more challenging to work on this species. However, because of its great importance, the complete genome of *P. vivax* was one of the first malaria parasite genomes to be sequenced and was completed in 2008 (Carlton et al., 2008; Bourgard et al., 2018), opening up many new approaches to studying the biology of the parasite for its control.

Plasmodium malariae

Plasmodium malariae (Figure 4C), or benign quartan malaria has a 3-day periodicity (again, remember the lack of zero when it was given this name by the Romans). Recognized by the ancient Greeks, it was not until the late 19th century that Golgi made careful note that there seemed to be 2 parasites infecting people—1 with fevers every 48 hours (that is, tertian malaria) and 1 that had a slightly different periodicity, which he correlated with slight differences in the parasites that he observed in the patients' blood (Garnham, 1966). *Plasmodium malariae* occurs throughout the world as well, but is most common in sub-Saharan Africa and the southwest Pacific though it can be very challenging to detect with just examination of blood films and thus molecular techniques such as PCR (polymerase chain reaction) are important to use (Garnham, 1966; Mueller et al., 2007).

In the early 1900s, a circus monkey was subjected to studies of its blood and a malaria parasite, similar in morphology to *Plasmodium malariae*, which was discovered and subsequently described as *P. brasilianum* (Garnham, 1966). These parasites were later observed in many wild monkeys in Central America and South America and for over a century were considered to be close relatives of—but not the same as—the human parasite. However, recent genetic results showed that these parasites were extremely similar (Fandeur et al., 2000), and in 2015, parasites that were genetically identical to *P. brasilianum* in wild howler monkeys were isolated from Indigenous Yanomami people living in Venezuela (Garnham, 1966; Lalremruata et al., 2015). When the complete genomes were sequenced, *P. malariae* and *P. brasilianum* were found to be the same species. A separate parasite, termed *P. malariae*-like, which was isolated from chimpanzees has since been found to be distinct (Rutledge et al., 2017).

Although *Plasmodium malariae* is typically considered a more benign form of malaria, it should not be dismissed as a public health concern. Because it can be difficult to diagnose with microscopy alone, it often goes undetected and may result in a fatal kidney disease (Eiam-Ong, 2003; Rutledge et al., 2017).

Plasmodium ovale

Until recently, *Plasmodium ovale* (Figure 6D) was generally considered to be the rarest form of the malaria parasites infecting humans. *Plasmodium ovale* has a rather scattered geographic distribution that primarily consists of western Africa, eastern Indonesia and New Guinea, and the Philippines, though of course due to the high mobility of humans, these parasites have also been reported in many other parts of the world (Mueller et al., 2007). Like *P. malariae*, *P. ovale* has also had a somewhat tumultuous taxonomic history. It was originally considered to be a variant of *P. vivax*, but was eventually described as a distinct species and named for the oval shape that some infected erythrocytes assume (Collins and Jeffery, 2005). Recently it was further split into 2 nominal subspecies on the basis of genetic data, *P. o. wallikeri*, and *P. o. curtisi* (see Sutherland et al., 2010).

Plasmodium knowlesi

In 2004, after a large number of malaria cases in Malaysian Borneo that were thought to have been *Plasmodium malariae* failed to amplify with species-specific primers, additional genetic testing confirmed that they were, in

fact, naturally acquired infections of *P. knowlesi*, a parasite thought to be confined to macaques (Singh et al., 2004). *Plasmodium knowlesi* was later reported from mainland Malaysia as well as in isolated cases in Thailand and in China, though likely the latter was acquired in Myanmar (Singh et al., 2004; Cox-Singh et al., 2008). It has the shortest of periodicities of the human parasites, completing a cycle in just 24 hours and can reach extremely high, even fatal, human parasitemias and so proper diagnosis of this species and distinguishing it from *P. malariae* is very important (Cox-Singh et al., 2008).

Malaria Parasites in Apes

In the early part of the 20th century, parasitologists working in western Africa discovered 3 species of *Plasmodium* infecting both wild chimpanzees and gorillas. Although there were similarities to the species known to infect humans, distinct names were given to these taxa nonetheless. In 1 of these cases, *P. reichenowi*, genetic material was available as the parasite was isolated and cultured from a chimpanzee that had been imported into the United States. When molecular systematic analyses using parasite DNA were first attempted, the resulting phylogenetic trees supported the idea that *P. reichenowi* was closely related to *P. falciparum*, but nonetheless was a distinct species (Escalante et al., 1998; Perkins and Schall, 2001). However, the larger picture of malaria parasites in apes was largely unknown until around 2010. Understanding of malaria parasites in apes began to change during this period, as new samples were collected, first from captive apes and then via the screening of a large number of non-invasively collected ape fecal samples. These results showed that there were many genetically divergent malaria parasite lineages present in African chimpanzees and in western gorillas (though interestingly, never in bonobos nor eastern gorillas even though these host species were very well sampled; Liu et al., 2010). A total of 4 species of ape malaria parasite have now been named that appear to be close relatives. The phylogenetic relationships amongst the 6 species of *Plasmodium* (*Laverania*) suggest that these parasites have shifted among human, gorilla, and chimpanzee hosts several times, although previous transferal experiments had suggested that they were largely host specific. The possibility that wild ape malaria parasites might be able to jump into human hosts as zoonoses is worrisome not only to public health officials, who see the apes as a large host population that is not treatable and may represent a reservoir of the parasites, but also to conservation biologists, who are concerned that parasites that have undergone selective pressure in humans might be more virulent in the wild ape hosts.

Impact on Human Genetics

Because of the enormous impact on human health, it is not at all surprising that malaria has served as an important selective force throughout our history and in fact, the disease is thought to have been the strongest source of natural selection on human evolution at least in recent times (Kwiatkowski, 2005). Two prominent examples are often discussed, sickle cell anemia and Duffy coat receptors.

Sickle Cell Anemia

The primary molecule inside red blood cells—and in fact, the only significant protein inside mammalian red blood cells—is hemoglobin. This molecule is made up of 4 chains of amino acids with an iron group in the center. Hemoglobin is adept at binding to the 2 key molecules of aerobic respiration, oxygen and carbon dioxide, and serves as the transporter of these gases throughout the bloodstream of vertebrates. Mutations in the hemoglobin molecule have been identified that alter its function. One of these, known as HbS, can disrupt the structure of the red blood cell, making it fragile and likely to collapse into a sort of sickle shape as opposed to the normal round shape if the tension of the respiratory gases is abnormal. If a person has 2 copies of the hemoglobin gene with this mutation, they will suffer from sickle cell anemia, a painful, largely untreatable, and sometimes fatal condition. One would predict, therefore, that natural selection would have removed these alleles from the human genome. And yet, they persist to this day. The reason is that people who are heterozygous for the variant hemoglobin gene have about a 10-fold higher protection from the forms of malaria infection most likely to cause death (Allison, 1954; Ackerman et al., 2005). If a red blood cell of a person who is heterozygous for sickle cell is infected with *P. falciparum*, the S type hemoglobin polymerizes, which then stalls the growth of the parasites (Archer et al., 2018).

Duffy coat Receptors

In human populations native to sub-Saharan Africa, there has been an almost complete fixation of a mutation that causes red blood cells to not express a protein that is necessary for the merozoite stage of *Plasmodium vivax* parasites to invade them (Kwiatkowski, 2005). This is known as the Duffy blood group-negative phenotype and makes those who have it essentially immune from *P. vivax* and *P. knowlesi*-caused forms of malaria.

Fighting Malaria as a Disease: Nets and Drugs

When Jesuit priests returned from missions in South America in the early 17th century, they brought bark from trees that indigenous Americans chewed to prevent shivering

and other ailments, hypothesizing that it might also be helpful with the shivering that accompanied the fevers of malaria (Meshnick and Dobson, 2001). It did have some success in treating malaria patients (remember that at this point, they still did not know exactly how the disease was transmitted from person to person) and became widely known in Europe as the fever tree, Jesuits' bark, or Peruvian bark. Around 1820, French chemists successfully extracted the main chemical component of the bark—quinine. Explorers searched the New World for trees that produced the highest concentrations of quinine and eventually seeds of *Cinchona ledgeriana* were used to start large plantations in Indonesia by the Dutch, and they controlled most of the world's production of quinine. (An interesting side note is that because of its bitterness, it was often mixed with spirits to make it more palatable—this may have inspired the gin and tonic cocktail!)

Synthetic antimalarial drugs, particularly the drug chloroquine, became widely used especially in World War II. Chloroquine was very effective; it worked by disrupting the parasite's ability to break down hemoglobin in the host cell and it was so widely used that in fact at one point it was sometimes mixed with table salt for mass distribution in malaria-endemic parts of the world. However, by the early 1960s *Plasmodium falciparum* parasites evolved resistance to chloroquine and the resistance quickly swept throughout most of the world. Mefloquine, sometimes known as Lariam®, and atovoquinone, often referred to as Malarone®, are 2 commonly used drugs that travelers to malarious parts of the world might be prescribed, though they are not safe to take for long periods of time (thus not usable for those people that live in malaria-endemic regions) and the parasites have evolved resistance to these compounds as well. In fact, there is not an anti-malarial drug that has been found or synthesized that the parasites have not evolved resistance to (Haldar et al., 2018). A compound known as qinghaosu or artemisinin (and its derivatives) that was used for centuries in China is now being produced and marketed, particularly in regions of Asia where parasites have evolved resistance to most of the other common anti-malarial compounds. It is a very effective drug and can even help patients in which the parasites have begun to sequester in capillaries. In most uses, it is administered along with other antimalarial drugs that have longer half lives in the body in an approach known as artemisin-combination therapy (White, 2008).

The other side of the malaria control coin is preventing people from acquiring the parasites in the first place, by stopping them from getting bitten by the mosquito vectors. This has been attempted through 3 main tactics: 1) Spraying insecticides, 2) poisoning the water sources where mosquito larvae are found, and 3) encouraging people, particularly children, to sleep under bed nets (Figure 7). All of these have had challenges or risks. In the 1950s there was a massive campaign to spray the insecticide DDT as a means of controlling mosquitoes and other insects. Although it worked very well to decrease mosquito populations, scientists quickly learned that this chemical exhibited bioaccumulation, or increased concentration as it moved up the food chain, ultimately being banned as a substance in the United States because of its severe environmental consequences. And, like the antimalarial drugs, the mosquitoes often quickly evolve resistance to these insecticides, causing them to lose their effectiveness (Hemingway et al., 2016). It is clear that combinations of methods and coordinated public health programs are going to have to be employed if malaria cases are going to be controlled, let alone if there is any hope of eradication of the disease. Some have argued that in order to be successful, evolutionary theory will need to be deployed into models of malaria control because these rapidly reproducing insects can more readily adapt to human-made chemicals than can be discovered and brought market (Read et al., 2009; Hemingway et al., 2016).

Figure 7. Children sleeping under a bed net. Ambitious programs to distribute insecticide-treated bed nets in malaria endemic areas has resulted in many fewer deaths in the past decade. Source: United States Global Health Initiative, 2006, https://commons.wikimedia.org/wiki/File:Malaria_prevention-Insecticide_treated_bed_net-PMI.jpg. Public domain.

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11

Protozoa

Trypanosomatidae

Trypanosoma (Genus)

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Phylum Euglenozoa

Class Kinetoplastea

Order Trypanosomatida

Family Trypanosomatidae

Genus *Trypanosoma*

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Chapter 11

Trypanosoma (Genus)

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Introduction

It is difficult to know exactly where to begin the introduction of the flagellated eukaryotic parasites that are classified in the genus *Trypanosoma*. This is because there is so much that can be learned about parasitism from the study of the morphological characters of these organisms; or by studies of how their populations cycle through individual mammals; or how they grow and reproduce in their insect vectors; or how these parasites are maintained in populations of mammals and their arthropod vectors; there is even a species that jumps from host to host without the inconvenience of having to use a vector; it has evolved past the need for a vector and instead uses the behavior of its infected host to transfer to a new potential host. However, starting off with a general classification of the group is generally a good idea, so that students who are just beginning, or even an advanced student, can see where the parasite belongs in the general scheme of life.

The trypanosomes are a **monophyletic** group (meaning, having a common origin from a single ancestral species) of single-celled eukaryotes (**eu** = true, **karyon** = nucleus; Greek), or those organisms that have a true nucleus. Obligate parasites of the genus *Trypanosoma* are included within the superorder Kinetoplastida. One of the current classifications looks like this:

Domain Eukaryota
Phylum Euglenozoa
Class Kinetoplastida
Order Trypanosomatida
Family Trypanosomatidae
Genus *Trypanosoma*

As seen in the list above, all known trypanosomatids are classified in the order Trypanosomatida, family Trypanosomatidae (see Moreira et al., 2004).

Members of this unique family are characterized by the single celled organism having an elongated cell body containing one flagellum emerging at the anterior part of the parasite and a single mitochondrion that is distributed throughout the cell body (see Figure 1 for details about the general morphology). The compressed DNA of this mitochondrion is called a **kinetoplast**, which is composed of **maxi-circles** and **mini-circles**, and is situated close to the base of the **flagellar pocket** (the place in the body from which the flagellum originates). Maxi-circles encode the genes required to perform the physiological function of oxidative phosphorylation that occurs, for example, in the midgut of the tsetse fly, and mini-circles are responsible for mRNA editing (Shlomai, 2004).

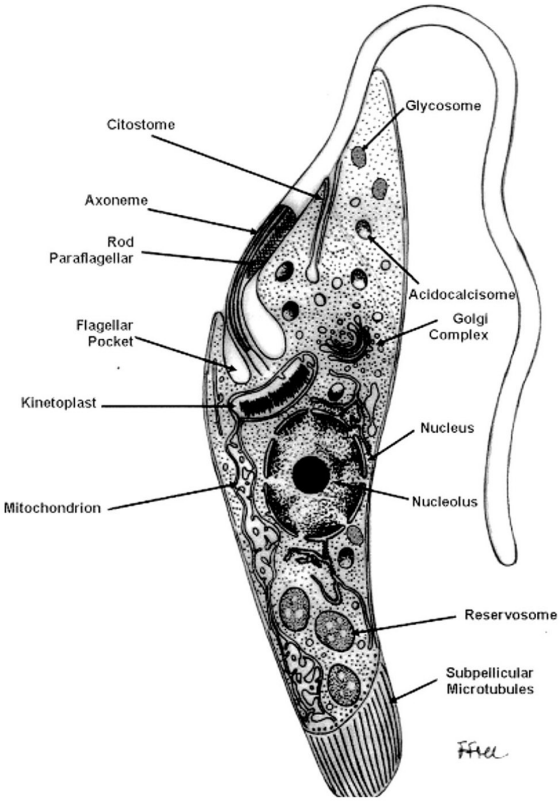

Figure 1. Schematic drawing, based on information obtained with the transmission electron microscope, showing the various structures found in the epimastigote form of *Trypanosoma cruzi*. Source of diagram: Souza, 1999. License: CC BY 4.0.

While species included in the family Trypanosomatidae have variable and distinct life histories, all species are obligate parasites. This family includes predominantly **monoxenic** forms (meaning, living on one host species), but also includes 4 genera with **heteroxenic** species (meaning, living in more than one host species) that are parasites of: 1) Plants (*Phytomonas* spp.); 2) sloths (*Endotrypanum* spp.); 3) other mammals and lizards (*Leishmania* spp.); and 4) all vertebrate classes (*Trypanosoma* spp.). Relatively recently, a fifth genus was proposed (*Porcisia* sp.) to include Neotropical porcupine-infecting species, previously known as *Leishmania hertigi* and *L. deane* (see Espinosa et al., 2018).

Concerning the monoxenic trypanosomatids, currently from 14 to 17 genera of parasites have been recognized, including those parasitizing Diptera, Hymenoptera, Siphonaptera, and Hemiptera. These monoxenic forms are distributed among the genera: *Angomonas*, *Blechomonas*, *Blastocrithidia*, *Crithidia*, *Herpetomonas*, *Kentomonas*, *Leptomonas*, *Lotmaria*, *Novymonas*, *Paratrypanosoma*, *Sergeia*, *Strigomonas*, *Wallacemonas*, and *Zelonia* (see Kaufer et al., 2017), as well as *Jaeinimonas, Lafontella*, and *Rhynchoidomonas* (see D'Ávila-Levy et al., 2015). Although described as monoxenic insect parasites, this trait (occurring in insects) seems not to be strict, since some of them have been reported parasitizing mammals, probably after being transmitted by insects. In fact, co-infections of *Leptomonas seymouri* and *Leishmania donovani* have been reported in patients with visceral leishmaniasis (Ghosh et al., 2012), and records of concomitant infections by *Leptomonas* sp. and *Herpetomonas samuelpessoai* in a HIV-positive human patient (Morio et al., 2008). In addition, there are records of *Herpetomonas* species infecting plants (Borghesan et al., 2013), and reports of *Blastocrithidia* sp. and *Chritidia mellificae* occurring in bats (Hodo et al., 2016; Rangel et al., unpublished data). Interestingly, when experimentally injected in the scent glands of opossums, *Leptomonas* sp. and *Chritidia* sp. not only established the infection, but also multiplied. The parasitism of scent glands of *Didelphis* spp. by monoxenic trypanosomatids was interpreted by Deane et al. (1984) as being the stepping-stone of trypanosomatids on their way to adapt to a mammal host.

Species of the genus *Trypanosoma* are parasites of vertebrates that are, with a few exceptions, transmitted among vertebrates by invertebrate vector hosts (Figure 2). Four primary morphological stages are recognized among trypanosomes: The extracellular trypomastigote, epimastigote, and spheromastiogote forms, and the intracellular amastigote form. These morphological forms occur in various stages among trypanosomatids with different species sometimes manifesting these forms differently. In 1972, the British protozoologist Cecil Hoare, in his foundational monograph, proposed the separation of the genus *Trypanosoma* in 2 **sections** based on the **transmission mode** of these parasites: **Stercoraria** and **Salivaria** (see Hoare, 1972). The divergence between these 2 sections would have occurred in the Cretaceous period (approximately 100 Ma = million years ago), accompanying the breakup of Gondwanaland and the separation of Africa from South America, Antarctica, and Australia. According to this classification that was widely accepted and used up to the current time, there are 3 sub-genera in **Stercoraria section**: *T.* (*Herpetosoma*) sp., *T.* (*Megatrypanum*) sp., and *T.* (*Schizotrypanum*) sp.; and 4 in **Salivaria section**: *T.* (*Dutonella*) sp., *T.* (*Nannomonas*) sp., *T.* (*Trypanozon*) sp., and *T.* (*Pycnomonas*). An eighth subgenus, *Tejeraia*, was proposed at a later date to include *Trypanosoma rangeli,* a South American parasite previously included in the *Herpetosoma* subgenus, but, as it later became clear, it is in fact transmitted by several hematophagous triatomine species (Añez, 1982).

The recent molecular tools with their great discrimination power that have been developed in the last decades have resulted in extensive revisions and descriptions of new genera and species. In fact, classifications up to 3 decades ago were made based on parasite morphology combined with the host species in which trypanosomatids were found. Another important point is the attention that was classically given to trypanosomatids with enzootic potential or potential to affect animals of economic interest. The current awareness of the importance of biodiversity (and parasites as important components of this) has widened the focus of attention in order to increase the interest in parasites not necessarily of humans or of domestic animals (Gardner and Campbell, 1992; Poulin, 2014; Jenkins et al., 2015).

Section Salivaria

Trypanosomes included in this section are highly prevalent in sub-Saharan Africa in the so termed Tsetse-Belt region and represent important health threats for humans and other animals with concomitant economic impacts in the areas of occurrence. Several species of the section Salivaria occur and can be found being transmitted in several countries beyond the African continent and the possibility of even greater geographic dispersion should not be neglected (Osório et al., 2008; Aregawi et al., 2019). Transmission occurs through inoculation of infective metacyclic forms as the insect vector feeds on blood of an infected mammal. For most species of salivarian trypanosomes but one, the only proven insect-vectors are various species of the hematophagous flies in the genus *Glossina* (the infamous and notorious tsetse flies of Africa). The only species that can be transmitted mechanically by other dipetera is *T. vivax*. *Glossina* spp. (Diptera: Glossinidae) (Figures 3 and 4) are the biological vectors of all salivarian trypanosomes and an individual fly may harbor

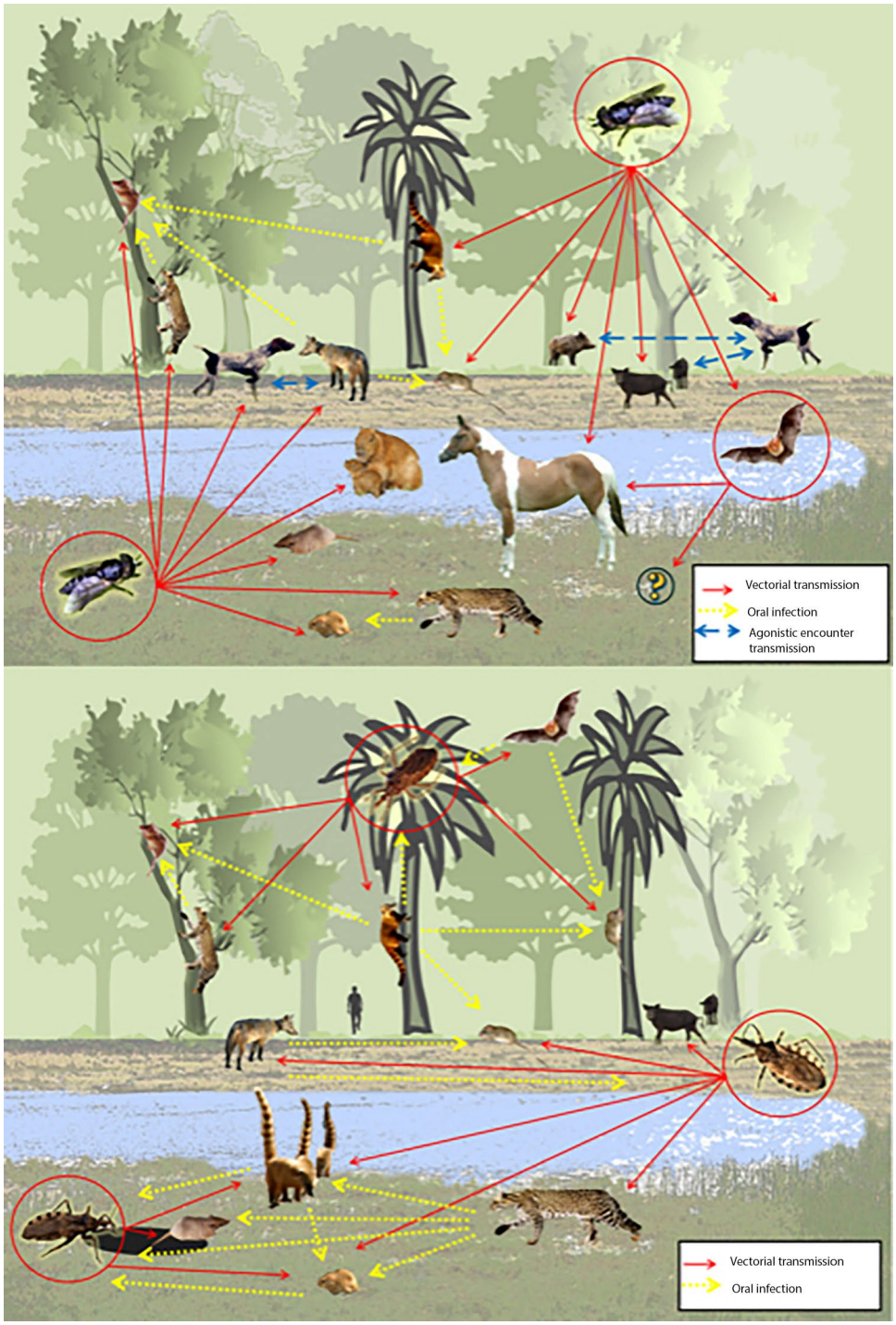

Figure 2. The wild and synanthropic reservoirs of *Leishmania* species represented graphically. Source: Rodrigues Roque and Jansen, 2014. License: CC BY-NC-SA 4.0 International.

more than one trypanosome species (Van den Bossche et al., 2004). All of the approximately 33 known species of *Glossina* that have been tested are able to act as vectors of salivarian trypanosomes; However, in contrast to many species of dipteran vectors, such as the blood-feeding Tabanidae, in which only females take blood meals, both sexes of *Glossina* are hematophagous and are capable of transmitting trypanosomes via saliva during blood feeding. Moreover, *Trypanosoma vivax* (another species of Salivarian trypanosome that lives in African mammals) can be transmitted by *Glossina* as well as by other hematophagous diptera (Fetene et al., 2021). Interestingly, *Trypanosoma evansi* does not appear to

Figure 3. A female tsetse fly (*Glossina morsitans morsitans*) from the Serap Aksoy Lab colony at Yale University School of Public Health. Tsteste flies transmit African trypanosomiasis. (Note: Taken by Geoffrey M. Attardo, this photo was a winner of the 2010 Fogarty Grantee Photo Contest, Fogarty International Center, United States, National Institutes of Health.) Source: G. M. Attardo, 2010. License: CC BY-NC-SA 4.0.

be transmitted by *Glossina* spp. and is mechanically transmitted from ungulate to ungulate by tabanids (order Diptera: family Tabanidae) and other hematophagous vector insects such as species of *Stomoxys*, *Atylotus*, *Lyperosia* (see Brun et al., 1998), and other slash and bite blood feeders such as *Desmodus rotundus* (common vampire bat) or *Diaemus youngi* the white-winged vampire bat in the Americas. *Trypanosoma equiperdum* the causative agent of Dourine in horses is a venereally-transmitted trypanosome and is generally considered to have a cosmopolitan (worldwide) distribution with the parasite generally absent in North America (north of Mexico), Australia, and western Europe (Brun et al., 1998; Gizaw et al., 2017).

The development of salivarian trypanosomes in the tsetse fly may be generally complex among *Trypanosoma* species and may be influenced by the insect immune response modulated by the fly's gut and symbiotic microbiota. Sexual reproduction in trypaonsomes has been reported, but only when the protozoans are actively reproducing within the insect vector-hosts (Gibson, 2015). Three interaction patterns of trypanosomes in the tsetse fly are recognized:

1) *Trypanosoma vivax* group (*Dutonella* subgenus) includes the trypanosomes with the lowest degree of interaction with the insect vector. This species is found across the Tsetse-Belt in Africa as well as in several countries in Latin America, occurring in wild and domestic animals. Recorded hosts for *T. vivax* include water buffalo, cattle, dogs, dromedary camels, horses, suids, and small ruminants. As noted, this species also occurs in wild animals that can serve as reserviors of infection for domestic animals. In this species development of the flagellates in the insect is restricted to the proboscis and cibarium where the parasite passes through 2 forms including both the **epimastigote** and **trypomastigote** stage. The entire life cycle of this species in the tsetse fly-vector is completed in as little as 3 days after initial infection. Forms of *T. vivax* in the blood stream of mammals are only of the monomorphic **trypomastigote** type. Representatives of this subgenus have evolved independently of tsetse flies and have adapted to mechanical transmission as noted earlier.
2) *Trypanosoma congolense* group *(Nannomonas* subgenus). Species included in this subgenus use a larger area of the digestive tract of the tsetse fly relative to species in the *Dutonnela* subgenus: In the gut, the ingested blood trypomastigotes differentiate into long trypomastigotes that migrate to cibarium and the proboscis of the flies where they differentiate into epimastigotes and, then, into metacyclic forms. Like *T. vivax*, mammalian blood stream trypomastigotes are also monomorphic.
3) Except for *T. evansi* and *T. equiperdum*, the representative of *T. brucei* group (*Trypanozoon* subgenus) are pleomorphic and go through a much more complex cycle in the vector. There are 2 proliferative forms in the fly, procyclic trypomastigotes in the gut and epimastigotes in the salivary gland and the entire life cycle can be completed in as little as 3 weeks (Sharma et al., 2009).

Salivarian trypanosomes may be highly pathogenic and lethal for their mammalian hosts, but a wide range of host species are tolerant to several of these trypanosome species. This is the case of native cattle breeds N'Dama and the West African shorthorn (WASH) (Ganyo et al., 2018). The basis of

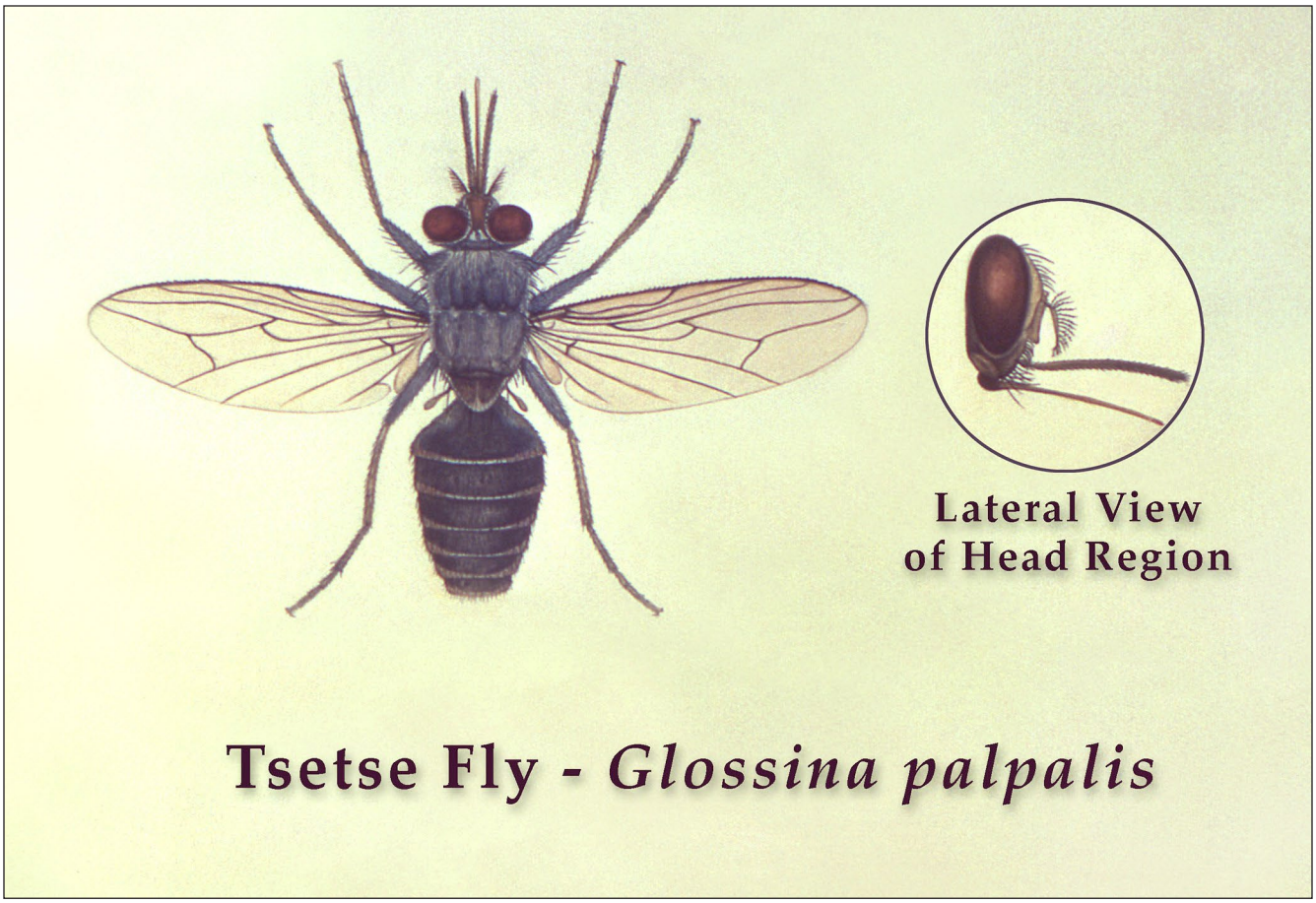

Figure 4. Tsetse fly, *Glossina palpalis*, top view with lateral view of head region. Source: United States Public Health Image library, image 17638; R. Darsie, 1976. Public domain.

this tolerance, although much studied with several hypotheses already formulated, is still a controversial subject. One unquestionable point is that whether the animals are trypanotolerant and trypanosusceptible is defined by their capacity to control anemia, which is the major outcome of the infection, and that determines whether or not host-animals remain competitive and productive.

The salivarian trypanosomes are not able to invade cells, and only extracellular trypomastigote and epimastigote forms are recognized. Trypomastigote forms are maintained in blood and body fluids of the infected mammal host. In the trypomastigote forms, 3 characteristics can be highlighted:

1) Two main types of blood trypomastigotes can be observed in most of the parasites from this section, including: a) Thin or slender forms are the dividing stage that are completely adapted to the mammal host, and b) broad or stumpy forms do not divide and possess well-developed mitochondria throughout the cell, and are adapted to the cycle in the vector dipterous host.
2) In the mammal host, where access to glucose is unrestricted, the mitochondrion is reduced in size and complexity and the parasite performs compartmentalized glycolysis, that is, within glycosomes. In this organelle, glucose is broken down into pyruvate and ATP, which is very effective whereas in the vector, where glucose is much more scarce, the now active mitochondria carries out an oxidative catabolism cycle generating CO_2, H_2O, and ATP oxidative cycle that results in CO_2 and H_2O; which is a more efficient mode of metabolizing glucose. Recently, it was shown that blood forms of *Trypanosoma brucei* are able to perform gluconeogenesis using glycerol as substrate for ATP production. The implications of this metabolic pathway are still unknown, but it demonstrates an important physiological plasticity. This pathway was suggested as being related to the passage of the parasite through tissues of its multiple host species (Kovařová et al., 2018). In the same way, gluconeogenesis was proposed as an important ATP source and is used by the procyclic forms in the midgut of the tsetse fly vector. In this environment, the parasite uses proline as substrate (Wargnies et al., 2018).

3) The ability of trypanosomes to sequentially modify their surface glycoproteins is an efficient escape mechanism from the mammalian host immune system. This variation in the surface coat of the protozoan are known as **VSGs** (**Variable Surface Glycoproteins**) initially described as **VATs (Variable Antigen Types)** (Barry et al., 1979). Salivarian trypanosomes live an extracellular existence in the blood and lymphatic system of the mammalian host and, are therefore, completely exposed to the humoral immune response, these parasites evolved to periodically alter all of their surface glycoproteins, as if changing a coat, in an efficient programmed mechanism where only one VSG is expressed at a time and is never repeated. It is estimated that there are about 10 million molecules anchored on the surface of these trypanosomatids being periodically exchanged, and about 2,000 vsg encoding genes regulating this process (Mugnier et al., 2016; Romero-Meza and Mugnier, 2020). This phenomenon is responsible for the parasitemia waves characteristic of mammals infected by salivarian trypanosomes. Also, their high motility, capacity of supporting non-immune defense mechanisms, and the mechanical forces inherent to blood circulation are important to survival in the mammal host (Stijlemans et al., 2016). The metacyclic forms of *T. brucei* develop in the salivary glands of the tsetse flies and also synthesize VSGs that; however, differ from the VSGs from the blood trypomastigotes (Kolev et al., 2017; Romero-Meza and Mugnier, 2020).

Tsetse flies live in moist savannah and woodlands, regions of more than 500 mm of rain a year, which include more than 30 countries across Africa (Cecchi et al., 2015). In those areas, human sleeping sickness is well-known and is caused by *Trypanosoma brucei rhodensiense* while *T. b. gambiense* causes Nagana, the non-human disease that is generally fatal in livestock and cycles in wild mammals (Büscher et al., 2017).

Humans are protected from infection by most of the African trypanosomes because of their production of a trypanolytic protein named Apolipoprotein 1 (APOL1), which is secreted by 2 protein complexes (TLF-1 and TLF-2) leading the formation of pores in the parasite membrane, resulting in its lysis. However, the 2 subspecies of *Trypanosoma brucei* associated with human sleeping sickness produce substances that are capable of lysing APOL1, the RAS factor in *T. b. rhodesiense* and TgsGP in *T. b. gambiense* (Capewell et al., 2015). Interestingly, baboons and one African human population named G1/G2 present a mutation in APOL1 that confers resistance to RAS and, therefore, to the infection by *T. b. rhodesiense*. The rare cases of humans that become infected with *T. b. brucei* and other species of trypanosomes are usually associated with people with some mutations that result in the absence of APOL1 production.

The genetic exchange that occurs solely in the insect vector of the salivarian trypanosomes, besides their huge repertoire of surface antigens, implies that new genotypes of salivarian trypanosomes may emerge to infect humans, and domestic and wild animals, and pose an important and worldwide health risk that would be magnified due to the absence of vaccines (Gibson, 2015).

Trypanosoma (Nanomonnas) congolense

Nannomonas trypanosomes include species that infect wild and domestic suidae (*Trypanosoma simiae* and *T. godfreyi)*, and *T. congolense*, a parasite that infects a broad spectrum of domestic and wild mammalian species and is the main cause of Nagana in Africa (Hamill et al., 2013; Morrison et al., 2016). These parasites are restricted to areas of occurrence of tsetse flies in sub-Saharan Africa and are described as extremely pathogenic for mammals (Cecchi et al., 2015). Some African breeds (Djallonke sheep, N'Dama cattle, and West African dwarf goats) are trypanotolerant, meaning that they are able to support the infection without anti-therapy and still maintain good health. The mechanisms underlying this tolerance probably depend on a genetic basis but this is still under debate (Yaro et al., 2016).

The trypomastigote forms are characterized by a flagellum that runs through the body of the parasite with a very short free end. The infection often starts with a skin lesion, a canker, where the parasites multiply, before reaching the bloodstream and lymphatic vessels of the host. In the vector, the multiplication takes place throughout the digestive tract before migration to the salivary gland, where the differentiation to metacyclic forms occurs (Dyer et al., 2013).

Human infection by *Trypanosoma congolense* has been only rarely reported (Truc, 1996). Compared to other African trypanosomes that infect livestock, *T. congolense* is considered as the most pathogenic, most prevalent, and most widely distributed trypanosomatid within the area of occurrence of *Glossina* spp. Three different genotypes of *T. congolense* are recognized: 1) The genotype Savannah, which is the most pathogenic and can affect a greater diversity of hosts, including carnivores; 2) the genotype Forest, described as poorly pathogenic and more often observed in cattle, goats, pigs, and dogs; and 3) the genotype Kilifi, considered as non-pathogenic and found infecting domestic ruminants (Rodrigues et al., 2014).

Trypanosoma (Trypanozoon) brucei

Trypanosoma brucei is the etiological agent of sleeping sickness in Africa and one of the few parasites able to cross the human blood-brain barrier, resulting in the nervous symptomatology observed in human disease. This parasite displays 3 subspecies: 1) *T. b. gambiense*, present in West Africa, associated with the more chronic form of human sleeping sickness, reported as less pathogenic; 2) *T. b. rhodensiense*, present in East Africa, associated with the acute form, the most pathogenic form of human disease; and 3) *T. b. brucei*, which infects wild and livestock animals, is associated with Nagana and is only rarely associated with human disease (Büscher et al., 2017).

The initial and recurrent symptoms of sleeping sickness are fever, tremors, muscle and joint pain, lymphadenopathy, malaise, weight loss, anemia, and thrombocytopenia. Later, neurological symptoms and meningoencephalitis can be present associated with mental retardation, convulsions, somnolence, and apathy that can progress to coma and death. This serious human disease is endemic in 36 African countries, including some epidemic areas in Angola, Democratic Republic of the Congo, and Sudan, and affects mainly people living in rural areas where tsetse fly is present. About 95% of human infections are caused by *Trypanosoma brucei gambiense* and commonly associated with *Glossina palpalis*. Those cases have a slower evolution of the disease and the infection can be present by months and even years without clinical signs. However, when the first signs appear, the disease is usually already in an advanced state and with the central nervous system compromised. The other 5% of the infections are caused by the *T. b. rhodensiense* and usually transmitted by *G. morsitans*. In these latter cases, the infection course is much faster and in a few months or even in a few weeks the disease progresses to the central nervous system presenting the characteristic neurological symptoms (Büscher et al., 2017).

In mammals, *Trypanosoma brucei* is always present in the extracellular trypomastigote forms and 2 morphotypes are recognized: 1) The slender forms, the divisionary forms that perform glycolysis in glycosomes and cannot survive in the insect vector; and 2) the stumpy forms, that do not divide in blood vessels, and display well developed mitochondrial ridges that are essential for survival in the vector.

Inside vectors, *Trypanosoma brucei* can colonize the whole digestive tract where both procyclic trypomastigotes and epimastigotes are present and can replicate themselves. The epimastigote forms that reach the salivary glands adhere to the microvilli of glands and perform cell division with subsequent differentiation to pre-metacyclic trypomastigotes, still attached to the salivary gland, but with fewer adhesion plaques. These pre-metacyclic trypomastigotes start to reacquire the coat of glycoproteins that are important in the blood phase of the infection. This form, named nascent metacyclic form, begins to detach itself from the glandular epithelium and will be totally free in the salivary gland when this differentiation is complete. At this point, the glycosomes are reverted to their spherical shape and the mitochondria are reduced to a small structure, which will no longer be functional while this parasite is in its mammalian host. The transmission will take place through the inoculation of these metacyclic trypomastigotes in the following blood meal (Vickerman et al., 1988; Dyer et al., 2013).

Trypanosoma (Dutonella) vivax

Trypanosoma vivax has a wide diversity of ungulate hosts; especially ruminants (order Artiodactyla). The infection occurs in Africa, Asia, Central America, and South America. Both wild and domestic ungulates (including buffaloes and antelopes); as well as equines and camels, are their most common hosts. Infection in laboratory animals has never been established (Osório et al., 2008). This parasite species can be transmitted both in cyclic and mechanical transmission. Cyclic transmission is reported in Africa, in areas where tsetse flies are present, and usually results in a more severe form of the disease. Mechanical transmission occurs in other parts of Africa (apart from the tsetse geographical area), in addition to Asia and the Americas, where the disease tends to be milder in cattle. In Brazil for example, outbreaks have always been associated with low mortality and few economic losses (Silva et al., 1996; Batista et al., 2007; Bastos et al., 2017).

In the mammalian host the trypomastigote forms are found exclusively in the blood. In *Glossina* spp., where the transmission is cyclic, this parasite differs in the replicative epimastigote forms that are restricted to earlier parts of the digestive tract. After the differentiation to trypomastigote forms, parasites migrate from the hypopharynx to the salivary gland where they differentiate into metacyclic trypomastigotes, which are the infective forms inoculated by the tsetse. Once in the salivary gland, *Trypanosoma vivax* can be maintained throughout the whole life of the vector. In mechanical transmission, there is no differentiation and the trypomastigote forms are carried from one mammal host to another by other hematophagous flies, especially those from the *Stomoxys* and *Tabanus* genera.

The first symptoms of *Trypanosoma vivax* infection are usually unspecific, such as anemia, fever, apathy, weight loss, and diarrhea. Diverse reproductive problems are associated with the infection, including transplacental transmission and abortion. When present, the neurological symptoms observed

are incoordination, muscle tremors, transient and/or permanent blindness, meningoencephalitis, and malacia (Batista et al., 2007; 2009).

Trypanosoma vivax was introduced into the Americas, probably with cattle brought from the European colonies in Africa. On this new continent, the parasite adapted to the mechanical transmission by several hematophagous insects, circulating among recently introduced domestic cattle and, perhaps, has spread among wild ungulates such as the cervids, never before exposed to this parasite. In Brazil, the first report of the parasite was in a buffalo in the swampy regions of Marajó Island, in the Brazilian Amazon (Shaw and Lainson, 1972). Later, in the late 1970s, this parasite was reported in sheep and cattle in the State of Amapá, also in Amazon region, and only a decade after this reported outside the northern region of the country, in cattle from the Brazilian Pantanal biome (Silva et al., 1996).

Possibly, the first outbreaks in the Pantanal were due to the increase of the displacement of animals from the north to the center-west of Brazil. In this biome, *Trypanosoma vivax* epidemiology is directly associated with drought and flood periods. In flooding periods, the reduction of pasture area increases the animal density per area, resulting in nutritional problems and resulting in higher susceptibility to infections, including trypanosomiasis (Silva et al., 1996).

Trypanosoma vivax can also be pathogenic for horses (da Silva et al., 2011). In both natural and experimental conditions, asinins were demonstrated to present high infection rates in subpatent and asymptomatic infections (Rodrigues et al., 2015). The infection of domestic ruminants by *T. vivax* results in severe economic losses, especially in South America. In spite of this, *T. vivax* remains poorly studied as a consequence of the inability to grow this trypanosome species in mice or in culture media.

Trypanosoma (Trypanozoon) evansi

The species *Trypanosoma evansi* masterfully exemplifies the genetic plasticity and consequent evolutionary success of salivarian trypanosomes. Together with *T. equiperdum*, *T. evansi* seems to have branched off from *T. brucei* due to the profound alterations of its kinetoplast DNA and, as a consequence, to have gained independence from a biological vector and to be transmitted mechanically, which has resulted in its enormous dispersion throughout Asia, the Americas, and Africa. The distinct kDNA alteration patterns observed in *T. evansi* samples suggest that *T. evansi* arose multiple times from a different *T. brucei* ancestor. These findings point to the necessity of revisiting the nomenclature of the members of the subgenus *Trypanozoon* (Radwanska et al., 2018).

Among all trypanosomes, *Trypanosoma evansi* is the one that is able to infect the largest variety of mammalian hosts, being dispersed in all continents (Desquesnes et al., 2013a). This parasite is the etiological agent of one of the major diseases affecting horses, called **Surra** (in the Old World) or **Mal de Caderas**, **Quebra Bunda**, or **Derrengadera** (in South America). The transmission is mechanical, being carried out normally by hematophagous flies from *Tabanus* spp. and *Stomoxys* spp. (Desquesnes et al., 2013b). The *Trypanosoma evansi* infection has been recently considered by OIE (World Organization for Animal Health) as a mandatory disease (Jaimes-Dueñez et al. 2017). Human cases are rare, but have been reported in Africa and Asia, usually associated with an extremely rare condition termed Tangier disease (Tomlinson et al., 1995).

Trypanosoma evansi belongs to the same *Trypanozoon* subgenus of the *T. brucei* species but is different from *T. brucei* whose transmission is totally dependent on cyclic transmission. *Trypanosoma evansi* is dependent only on mechanical transmission, even by tsetse flies. That is because, at some point on the evolutionary path of these parasites, *T. evansi* differentiated from *T. brucei* and lost the kDNA maxi-circles where most of the genes responsible for the oxidative metabolism and multiplication are located in *Glossina* spp., making *T. evansi* incapable of multiplying in the biological vector. Currently, some researchers suggest that this species comprises total or partial diskynetoplastic *T. brucei* strains. Although still considered to be a different species, some authors propose that *T. evansi* (and *T. equiperdum*) be classified as subspecies of *T. brucei,* or even variant strains of *T. b. brucei* (Carnes et al. 2015; Wen et al. 2016).

Trypanosoma evansi is a monomorphic parasite, and the trypomastigote form is the only recognized morphotype. Its trypomastigotes are the replicative forms observed exclusively in the blood of infected mammals and its morphology is exactly identical to the stumpy forms of *T. brucei*. Infection in mammals usually results in very high parasitemias, favoring mechanical transmission. Besides the mechanical transmission by hematophagous flies and the iatrogenic form through sharing of contaminated fomites, hematophagous bats display a differentiated feature, being able to act both as reservoir and vector of this parasite. That is because, in addition to being infected as all other mammalian hosts (which means that the parasite multiplies in the blood of these animals), it can still transmit the parasite that is easily present in its saliva during a blood meal (Desquesnes et al., 2013b). The oral route and agonistic encounters are also proposed as transmission routes in the wild (Herrera et al., 2011).

The estimated date of entry into South America by *Trypanosoma evansi* is still controversial. Although it is

hypothesized that *T. evansi* was introduced by infected horses of European colonizers (Desquesnes et al., 2013a), other authors propose that their introduction was much earlier, perhaps carried by the first primates and caviomorph rodents that came directly from Africa to South America, about 35–40 Ma (= million years ago). Caviomorph rodents, including capybaras, are considered to be important *T. evansi* reservoirs in the Pantanal region (Herrera et al., 2004) and infected rodents may have entered the Americas through the well described island-hopping or even sweepstakes dispersal processes (Lavocat, 1974; Raven and Axelrod, 1975; Flynn and Wyss, 1998).

In South America, trypanosomiasis caused by *Trypanosoma evansi* is economically very important in the flooded areas of the Brazilian Pantanal and Argentinean Chaco regions. These regions have a great concentration of livestock, and horses are essential for cattle handling. Outbreaks occur sporadically, though more common after a flood period, and result in a diversity of clinical features, from mild to severe fulminant forms (Herrera et al., 2004). In most severe cases, horses may present with anemia, emaciation, and subcutaneous edema of the lower body regions, with reports of abortion in pregnant females, and various types of neurological manifestations. The characteristic symptoms that inspired the name of the horse disease are the atrophy of the great muscular masses of the pelvic limbs, devolving to incoordination and ataxia (Desquesnes et al., 2013a).

The main wild reservoirs in the Pantanal region are the coatis and capybaras because they: 1) Are quite abundant in the region; 2) present high infection rates with long-lasting patent parasitemia; and 3) may remain infected for a long time. Capybaras support infection by *Trypanosoma evansi* without anemia and while maintaining good general health. Coatis, in contrast, when infected by *T. evansi* display anemia (Herrera et al., 2002; 2004). Infected horses may also act as reservoirs because the persistence of the parasite in asymptomatic animals after treatment is not rare (Herrera et al., 2004).

Usually reported in domestic animals around the world, studies on *Trypanosoma evansi* transmission in the wild are generally restricted to the Brazilian Pantanal. In this region, *T. evansi* can be considered a typical enzooty, with several infected wild mammals already found and occurring in sympatry with other trypanosomes, such as *T. cruzi* and *T. rangeli*. Interestingly, most infections observed in the wild seem to be subpatent and anemia is not commonly observed (Rademaker et al., 2009).

Trypanosoma (Trypanozoon) equiperdum

The other species within the *Trypanozoon* subgenus, also considered as a subspecies of *Trypanosoma brucei* or a mutant strain of *T. b. brucei* by some authors (Carnes et al., 2015; Wen et al., 2016), is *Trypanosoma equiperdum.* This species is the causative agent of **equine Dourine**, a disease that affects horses and other equidae. The transmission is exclusively venereal and the parasite is found only in genitals and their secretions. Asinines are usually reported as asymptomatic carriers of the parasite (Gizaw et al., 2017). Although as widespread as *Trypanosoma evansi* in the world, the reports of infection are quite intermittent and most studies are only case reports. The symptoms are similar to other trypanosomiases, such as fever, anemia, and emaciation, besides symptoms more specific to the genital organs, such as edema of the genitalia and mammary glands. More severe forms may progress to incoordination, facial paralysis, and death (Gizaw et al., 2017).

The difficulty in studying *Trypanosoma equiperdum* is that there are almost no available isolates of this parasite, and most isolates are from the beginning of the last century and lack essential information such as isolation site, year, and even host. After a long time without description of new isolates, in 2015, a group from Venezuela obtained the first 2 isolates of this parasite in Latin America (Sánchez et al., 2015). In 2016, another group obtained a new isolate from the urogenital tract of a horse in Mongolia (Suganuma et al., 2016). The lack of knowledge about *T. equiperdum* reinforces the question about the validity of this species (or subspecies).

Trypanosoma (Tejeraia) rangeli

One trypanosome that can be classified as neither Stercoraria nor as Salivaria**,** is *Trypanosoma rangeli* (see Grisard, 2002). Actually, *T. rangeli* is a parasite species transmitted by the saliva of its insect vector but shares numerous characteristics with other species of the Stercoraria section, as it is easily cultivated in axenic media (which is not observed in the Salivarian trypanosomes). It also shares mammalian hosts and vectors with *T. cruzi*. Currently, this parasite species is classified in the subgenus *Tejeraia* that was created in 1982 only to classify *Trypanosoma rangeli*, until then classified within the *Herpetosoma* subgenus, within the Stercoraria section (Añez, 1982).

Trypanosoma rangeli is a multi-host (mammal) parasite found exclusively in the Americas and is capable of infecting humans. The main vector species are triatomines from the *Rhodnius* genus, in which *T. rangeli* differentiates to the infective metacyclic forms in the salivary gland of the insect (Guhl and Vallejo, 2003). Moreover, other triatomine species may also maintain *T. rangeli*, as in the case of *Triatoma vitticeps* (see Dario et al., 2017a). The genetic diversity within *T. rangeli* was first observed based on the difference of a nucleotide sequence from its mini-circles, resulting in

the recognition of 2 separate populations, KP1(+) and KP1(−) (Vallejo et al., 2002). Further studies have shown that this genetic polymorphism is even more extensive and 5 lineages could be described, from A to E (Maia da Silva et al., 2009). It was first proposed that the divergence of these lineages was related to different *Rhodnius* species involved in the transmission, but it is now accepted that this separation of lineages is not strict. In fact, the characterization in distinct lineages is recent and we do not have enough sampling to propose associations between *T. rangeli* lineages and mammal hosts and/or ecotypes (Urrea et al., 2011; Dario et al., 2017a).

In the mammal host, only the blood trypomastigote form is observed and the current consensus in the scientific community is that *Trypanosoma rangeli* does not differentiate in amastigote forms, as occurs in *T. cruzi*. In the *Rhodnius* vector, the predominant and replicative form is the epimastigote that colonizes the vector's gut. Some of these parasites differentiate into trypomastigotes in the final portion of the intestine and are eliminated with the feces, but these forms are not infective for mammals. Most of the epimastigote forms invade the vector's hemocoel, where they can multiply both inside and outside hemocytes. From the hemocoel, some of these parasites reach the salivary gland and differentiate into metacyclic trypomastigotes that are transmitted through the saliva during a blood meal (Guhl and Vallejo, 2003).

Some points of this transmission cycle still need to be clarified. First, only the blood trypomastigote form is described in mammal hosts, which is considered to be non-replicative. Otherwise, this parasite is possibly able to multiply in the mammalian host because there are numerous cases of persistence of parasitism even after long periods after exposure. For instance, *Trypanosoma rangeli* was isolated from Brazilian patients who had been out of risk areas for many years and were being treated as having Chagas disease (de Sousa et al., 2008). These cases suggest that this parasite species can multiply in the mammal host, in a mechanism still not identified.

Another intriguing aspect is the niche occupied by *Trypanosoma rangeli* in mammal hosts. This parasite is always diagnosed in blood, but it has been isolated from bone marrow from an anteater in the Amazon region. The presence of *T. rangeli* in this unorthodox niche probably occurred through blood or lymph circulation, as described for other trypanosomes, and could had been influenced by the coinfection of *T. cruzi* and *Leishmania infantum* that were also diagnosed in the same host (De Araújo et al., 2013).

The *Trypanosoma rangeli* infection in mammals are considered innocuous or non-pathogenic, although due to the unknown mechanism of parasite multiplication in mammals, the discovery of *T. rangeli* in bone marrow and the isolation in Chagas disease patients are aspects that have to be considered in the endorsement of this assumption. On the other hand, the pathogenicity of *T. rangeli* infection in invertebrate hosts is well described and reports of the influence of the parasite load on insect molt and destruction of intestinal epithelium during parasite invasion to the hemocoel are some of the damaging effects usually observed in infected bugs (Ferreira et al., 2010; García et al., 2012).

Section Stercoraria

Trypanosomes of this section include numerous species of *Trypanosoma* that live in the intercellular spaces and/or in the blood of their mammalian hosts. Most of these do not display mechanisms of colonizing the intracellular environment or disguising immune response by using variable surface antigenic variation as observed in some salivarian trypanosomes. This is the case for several species of *Trypanosoma* from this section. Stercorarians include heteroxenous parasites that are transmitted between species belonging to all vertebrate classes and hematophagous invertebrates on all continents. Three main evolutionary stages are recognized in this section, including: 1) Extracellular trypomastigote forms, 2) extracellular epimastigote forms, and 3) intracellular amastigote forms. Interestingly, in the stercorarian trypanosomes, replication by cellular division/binary fission of these trypanosomes in mammals occurs only in either the amastigote or the epimastigote stages; fission of the trypomastigote forms in this group is unknown (Hoare, 1972).

All species from this section possess trypomastigote forms with flagella protruding anteriad of the cell with a large and non-terminal kinetoplast. In the vector insects, the final development of the parasites, which corresponds to the formation of metacyclic trypomastigotes, occurs in the posterior portion of the digestive tract of the insect vector host. The main characteristic, which gives the name to the section (stercoraria or posterior station), is their transmission method which is always via insects' feces by rubbing metacyclic trypomastigotes into a wound, eye mucus membranes, or oral mucus membranes (Hoare, 1972).

Trypanosoma (Herpetosoma) lewisi

The subgenus *Herpetosoma* comprises all the species described in the *Trypanosoma lewisi* group, which is the type species of the subgenus. Species from this subgenus include trypanosomes from many species of rodents, in addition to a lagomorph trypanosome called *T. nabiasi*. Most known vectors are fleas, but for many *Trypanosoma* (*Herpetosoma*) species, the transmission cycle is still completely unknown.

Trypanosoma lewisi is a cosmopolitan parasite of *Rattus* spp. This trypanosomatid species was probably introduced to the different continents and countries due to its association

with *Rattus rattus*, a synanthropic rodent that in turn has accompanied humans since their first voyages. *Trypanosoma lewisi* was previously considered specific to *Rattus* spp. and not capable of infecting the other cosmopolitan rodents (*Mus musculus*) in experimental conditions. Currently, it is recorded in some wild rodent species (Mafie et al., 2019), besides primates, including humans (de Sousa, 2014). The phylogenetic analysis of *T. lewisi* isolates from *Rattus* spp. and primates proposed that *T. lewisi* underwent a process of host switching between rodents and primates through accidental contamination of primates with infected fleas from these rodents (Maia da Silva et al., 2010). Some cases have been reported in Asia, associated with fever, anemia, and immunosuppression (Verma et al., 2011). Some authors consider *T. lewisi* a neglected re-emerging human pathogen (Lin et al., 2015) in spite of the rarity of cases of human infection by *T. lewisi* worldwide and because it always presents in immune incompetent individuals or people living in close contact with rats (Verma et al., 2011; Shah et al., 2011).

The recognized vectors of *Trypanosoma lewisi* are *Xenopsylla cheopis* and *Nosopsyllus* sp., but it is believed that other fleas, such as *Ctenocephalides canis*, *Leptopsylla segnis*, and *Pulex irritans*, can also act as vectors (Hoare, 1972). No cell invasion is reported in vertebrates, and the infection is considered innocuous in immunocompetent hosts, although it can be lethal in newborn rats or increase susceptibility to other parasites when presenting as a co-infection, as with *Toxoplasma gondii* (Ríos Carrera et al., 2009) or *Cryptococcus neoformans* (Gross et al., 2006).

Two stages of the parasite can be observed in a vertebrate's blood, the replicative epimastigote forms, and the trypomastigote forms. Inside the digestive tract of fleas, the trypomastigote forms invade the epithelial cells of the stomach and differentiate into amastigote forms, which are replicative, and differentiate again to trypomastigote forms before returning to the digestive lumen. After reaching the flea's midgut, the trypomastigote forms differentiate into replicative epimastigotes that will differentiate into metacyclic trypomastigotes at the end of the digestive tract. Besides the contaminative route, oral transmission through accidental ingestion of fleas has been demonstrated for *Trypanosoma lewisi* and other species from the same subgenus, namely, *T. microti*, *T. evotomys*, and *T. grosi* (see Maraghi et al., 1995).

In mammals, infection by *Trypanosoma* (*Herpetosoma*) species is characterized by intense and short parasitemias. The infection is easily controlled by the host in about 3 weeks due to a humoral immune response because the parasite has limited capacity for antigenic variation and does not invade mammal cells. A characteristic observed in *T. lewisi*, and also believed to occur in other species from the same subgenus, is the production of an IgG immunoglobulin named ablastin that inactivates the replicative forms of the parasite leading to the abrupt remission of the parasitemia. After this, rodents become resistant to new infections (Drew and Jenkin, 1982). The abrupt remission of parasitemia occurs only when preceded by an initial phase of infection establishment where, most probably, other factors are involved. The passive transfer of ablastin from one infected rodent to another was observed, which resulted in a partial control of the infection, but not as abruptly as observed in natural infections (Drew and Jenkin, 1982).

An intriguing aspect of the transmission cycle has been observed in *Trypanosoma musculi*, a parasite considered restricted to *Mus musculus*. After the phenomenon of ablastin, rodents infected by *T. musculi* become resistant to new infections and parasitemia is no longer observed, but throughout their life, the infected rodents still maintain some parasite forms, including replicative forms, in the vasa recta of the kidneys. These forms are biochemically and molecularly different from blood forms and appear to represent a new evolutionary stage of the parasite that are not inactivated by the host's immune system, due to the high concentration of urea (Monroy and Dusanic, 2000). The consequences of the existence of this distinct stage in the life cycle, the occurrence of this phenomenon in other *Herpetosoma* species, and the evolutionary impact of this parasite persistence are still unknown aspects.

Besides *Trypanosoma lewisi* and *T. musculi,* some other related species described in the same *Herpetosoma* subgenus are: *T. microti,* described in *Microtus* spp. and also directly transmitted through agonistic contact in the reproductive season; *T. evotomys* and *T. grosi* from Old World rodents; *T. kuseli* and *T. otospermophili* from squirrels; and *T. nabiasi,* a parasite species specific of the lagomorph *Oryctolagus cuniculus* present in the New World and Australia, the latter as a result of the human introduction of infected fleas in an attempt to control the rabbits (introduced one century before) with the Myxomatosis virus (Hamilton et al., 2005).

Trypanosoma (Megatrypanum) theileri

The morphology of parasites from the subgenus *Megatrypanum* is very typical: they have very large cells with a visible undulating flagellum that adheres to the entire body of the parasite. Trypanosomes from this subgenus are transmitted by a diversity of vectors, including hematophagous flies, fleas, ticks, and *Pseudolynchia* sp. This subgenus comprises species that infect a wide variety of hosts, including marsupials, rodents, primates, and, mainly, varieties of bovinae cattle (Kingston, 1991; Kelly et al., 2017). The species *Trypanosoma* (*Megatrypanum*) *theileri* is a hemoparasite found in

Artiodactyla species worldwide and is the type species of the subgenus *Megatrypanum*. Recently, a second escape mechanism of modeling the trypanosome cell surface, distinct from the VSG's coat of the salivarian trypanosomes, has been described. Actually, the surface of *Trypanosoma theileri* cells can be modeled by the expression of a mixture of proteins (Kelly et al., 2017).

The transmission cycle of *Trypanosoma theileri* is not completely elucidated yet. Mammals are proposed to have a replicative epimastigote form, but this parasite form is very rarely observed in field studies, probably because these forms quickly differentiate into trypomastigotes, which is the predominant morphological type detected in mammals' blood. In mammals, those trypomastigote forms are capable of invading all body fluids, in addition to some tissues, such as lymph nodes, kidney, spleen, and brain. For long time, it was accepted that *T. theileri* did not differentiate into amastigotes, but the existence of these stage forms was recently demonstrated in vitro, including the ability to differentiate and invade other cells in a process similar to that observed in *T. cruzi* (see Lee et al., 2013). In vectors, the epimastigote forms of the parasite replicate in the gut before differentiating into metacyclic trypomastigotes that are eliminated in the feces. The tabanids are the vectors of *T. theileri,* but a tick species (*Hyalomma anatolicum*) was proposed before as an alternative vector (Latif et al., 2004). As observed for other Stercorarian trypanosomes, oral transmission through accidental ingestion of infected vectors is proposed as an important infection route (Kelly et al., 2017).

Trypanosoma theileri is a cosmopolitan and opportunistic parasite. The parasitemia in bovines is usually associated with some unspecific clinical signs. Infection may remain subpatent and asymptomatic for several years but may result in high parasitaemia and pathogenicity when associated with other factors, such as stress, malnutrition, or pregnancy. This parasite species is recognized in bovines and buffaloes from South America, and at least 10 genotypes are recognized, most of them specific in terms of mammal species (García et al., 2011).

Besides *Trypanosoma theileri,* some other species from the same *Megatrypanum* subgenus are: *T. minasense* from New World primates; *T. melophagium* from sheep*; T. theodori* from goats; *T. cervi* and *T. mazamarum* from cervids; *T. freitasi* that colonize scent glands of *Didelphis* spp.; *T. samueli* and *T. saloboense*, from *Monodelphis* sp. from the Amazon region; *T. peba* from the 6-banded armadillo *Euphractus sexcinctus; T. legeri* from the anteater *Tamandua tetradactyla; Trypanosoma pestanai* isolated from European badgers; and *T. caimani* from alligators from the Brazilian Pantanal; as well as a high diversity of bird trypanosomes. For the less studied *Megatrypanum* species, other vectors (apart from tabanids) are involved in the transmission: keds (*Melophagus ovinus*) are involved in *T. melophagium* transmission (Gibson et al., 2010), *T. pestanai* is transmitted by fleas (Peirce and Neal, 1974; Lizundia et al., 2011), while *T. cervi* are also spread by keds (Böse and Petersen, 1991). It is worth mentioning that many of these parasite species were described based on morphology, before the employment of molecular techniques for parasite identification. As a result, some species may not be valid, as in the case of *T. saimirii* and *T. leeuwenhoeki,* currently recognized as synonyms of *T. rangeli* (See Stevens et al., 1999a; Ziccardi et al., 2005).

Trypanosoma (Schizotrypanum) spp. and Other Species Related to *T. cruzi*

Almost all the *Trypanosoma* (*Schizotrypanum*) species are parasites exclusive of bats. The exceptions are *T. cruzi,* a multihost parasite that may also infect bats, and *T. dionisii,* a parasite species considered specific to bats, but recently observed also in human cardiac tissue and in the marsupial *Monodelphis americana* (See Lima et al., 2015a; 2015b; Dario et al., 2016; 2017a; 2017b). Parasites from this subgenus are morphologically identical and all of them are able to differentiate into metacyclic forms and invade cells, where they differentiate into amastigotes.

Until the 1990s, most *Trypanosoma* infections in bats were described as *T. cruzi*-like because of the difficulties differentiating among *Schizotrypanum* species. Currently, due to advances of molecular techniques, especially since it is easier now to analyze DNA sequences, the variety of *Trypanosoma* species infecting bats has been shown to be enormous, and this diversity has been increasing annually with descriptions of new *Trypanosoma* species infecting bats. Some *Trypanosoma* species reported in bats are: *T. cruzi marinkellei* and *T. wauwau,* described in bats from Central America and South America; *T. vesperitilionis* and *T. dionisii,* described in bats from both the Old World and the New World; *T. pipistrelli* from Old World bats; *T. pteropi*, *T. hipposideri*, and *T. teixeirae* from Australian bats, including the enormous flying foxes; *T. hedricki* and *T. myoti* from bats surveyed in North America; *T. erneyi* and *T. livingstonei* from Africa; and yet others (Lima et al., 2012; 2013; 2015a; 2015b).

The majority of the descriptions of *Trypanosoma* infection derived from insectivorous bats suggest that, besides the vectorial contaminative route, oral infections must also occur (Lima et al., 2015a; 2015b; Dos Santos et al., 2018). The vectors involved in the majority of bat trypanosome transmission are unknown. *Trypanosoma cruzi marinkellei* is currently recognized as a subspecies of *T. cruzi* and reported to be transmitted only by triatomines of the genus *Cavernicola*,

associated with caves (Marinkelle, 1982). Vectors of *T. dionisii* and *T. vespertilionis* are reported to be cimicids, but both *T. c. marinkellei* and *T. dionisii* were recently found infecting *Triatoma vitticeps,* a triatomine bug very common in the Atlantic rainforest (Dario et al., 2017a). The close association of *Schizotrypanum* parasites and bats suggests a long coevolutionary process. However, the association with different vectors points to an independent evolution between species within this subgenus.

Other *Trypanosoma* species more recently described were not classified in any subgenus but are phylogenetically closer to *T. cruzi* and known as trypanosomes of the *T. cruzi* clade. Besides all the above mentioned trypanosomes of bats, this clade includes *T. noyesi,* a trypanosome described from the Australian endangered woylie (*Bettongia pencillata*); 2 isolates from African terrestrial mammals (1 feline and 1 primate); *T. conorhini*, a species taxonomically classified in the subgenus *Megatrypanum*, but phylogenetically in the *T. cruzi* clade; and *T. janseni,* a species recently described in Brazilian opossums that, along with *T. wauwau,* forms a sister group of the trypanosomes found in Australian marsupials (Hamilton et al., 2009; Botero et al., 2016; Lopes et al., 2018).

From these, one of the most studied is *Trypanosoma conorhini.* This species infects *Rattus rattus* a synanthropic rodent and the kissing bug *Triatoma rubrofasciatta* all over the world, and only experimentally was demonstrated to be able to infect *Mus musculus* and *Macaca mulatta* (Deane et al., 1986). The only parasite form observed in the rodent's blood is the trypomastigote, without evidence of cell invasion and differentiation to amastigote forms. The biological cycle in the vector includes the replication of the epimastigote forms in the bug's gut and differentiation in metacyclic trypomastigotes that are eliminated in the feces (Hoare, 1972). The fact that this trio–*Rattus rattus*, *Triatoma rubrofasciatta*, and *Trypanosoma conorhini*–are found together in different parts of the world indicate that their dispersal process occurred together, probably from Asia, which represents a unique joint migration process within the heteroxenous protozoa (Deane et al., 1986).

In general, the vectors involved in the transmission of most of the *Trypanosoma* species from the *T. cruzi* clade are unknown, but all of the vectors described up to now are hematophagous hemipterans. In fact, the knowledge of the diversity of *Trypanosoma* species has been a growing subject of study, especially in the past decade when DNA sequencing tools became more accessible. These studies have shown a much greater diversity than was previously known and that the evolutionary paths within this clade are still not fully understood.

Trypanosoma cruzi

Trypanosoma cruzi is the etiological agent of one of the most important and neglected parasitic disease, Chagas disease (Chagas, 1909). However, the human infection is only a minor trait of this parasite ecology that is a wild animal parasite maintained among dozens of species of mammals and triatomines (family Reduviidae, subfamily Triatomiane). In fact, *T. cruzi* is a multi-host parasite, able to infect virtually any mammal species and, within them, any nucleated cell (Jansen et al., 2015). In spite of presenting a population structure that is basically clonal, mitochondrial introgression events are not unusual and evidence of genetic exchange has already been described in *T. cruzi*. Also, recombinant strains have been described (Lewis et al., 2011).

There are 2 non-mutually exclusive hypotheses that explain the origin of *Trypanosoma cruzi*. The first, called **Southern Supercontinent**, places the origin of this parasite from an ancestral trypanosome of marsupials, which represented the dominant local fauna, in a supercontinent formed by South America, Antarctica, and Australia. This hypothesis is supported by: 1) The presence of a trypanosome related to *T. cruzi*, named *T. noyesi* (Botero et al., 2016), isolated from an Australian woylie (besides other related isolates from Australian marsupials); 2) the estimated divergence between *T. cruzi* and *T. brucei* (approximately 100 million years) that is the approximate time of separation of this supercontinent from Africa (Stevens et al., 1999b); and 3) the recent description of *T. janseni*, which was described in Brazilian opossums and clusters with *T. wauwau* in a well-supported clade, representing a sister group of the trypanosomes found in Australian marsupials (Lopes et al., 2018).

The second hypothesis points to the origin of *Trypanosoma cruzi* from ancestral *Trypanosoma* species of bats and is called the **Bat Seeding Hypothesis** (Hamilton et al., 2012). This hypothesis is supported mainly by: 1) The increasing evidence of the diversity and polyphyly of bat trypanosomes; and 2) the description of infection of trypanosomes from the *T. cruzi* clade in African monkeys and palm civets (which does not represent a single lineage that could have been introduced more recently; that is, post-separation of the continents) (Hamilton et al., 2009). Several processes of trypanosome spillover from bats to terrestrial mammals must have occurred until one of them was successfully established and the evolution of this parasite resulted in a new species currently known as *T. cruzi* (Hamilton et al., 2012).

The contaminative transmission of the parasite, described as the classic form of transmission, occurs when the insect vector eliminates metacyclic trypomastigote forms with feces during a blood meal. These parasites penetrate through lesioned skin or mucous membranes or when the person

scratches the location of the insect bite. In the mammal host, these parasites invade the nucleated cells, in which they differentiate into the replicative amastigote forms. A novel differentiation into blood trypomastigotes occurs before the cellular disruption that results in the release of these forms that can invade other cells or circulate in blood vessels and be ingested by other Triatominae in a new blood meal. In invertebrate hosts, *Trypanosoma cruzi* differentiates into the replicative epimastigote forms and only in the final portion of the insect gut, the parasites differentiate into the metacyclic trypomastigote forms that are the infective forms eliminated in the feces.

This classical route of transmission, however, does not reflect all possible routes of infection in mammals, including humans. From the 4 evolutionary stages of the parasite, only epimastigotes may not be infective, mainly when they are in the exponential growth phase. Moreover, epimastigotes of the stationary phase are already resistant to the complement system and already infective (Kessler et al., 2017). Nutrient impoverishment and starvation of the parasite acts as a trigger for metacyclogenesis (Barisón et al., 2017). Human infections can also occur during blood transfusion and organ transplantation, and these are the most important infection routes in non-endemic countries such as the United States, Spain, and Japan (Gascon et al., 2010). The congenital form, well characterized in humans, seems to have a more regionalized importance, especially in the southern region of South America and non-endemic areas. The oral route has emerged as a very important transmission route in the wild and has been responsible for an increasing number of human infections in the past few decades (Coura, 2015). In the South American Amazonian area, especially in Brazil, this transmission route represents more than 90% of new cases and is usually associated with intake of food contaminated by feces of infected triatomines or infected vectors accidentally crushed along with food, such as sugar cane and açaí juice (Figure 5) (PAHO, 2009). This transmission route requires specific surveillance measures whose operation is still not completely known (Xavier et al., 2014).

Figure 5. The oral route has emerged as a very important transmission route in the wild and has been responsible for an increasing number of human infections in the past few decades. In Amazonia, especially Brazil, this transmission route represents more than 90% of new cases and is usually associated with intake of food contaminated by feces of infected triatomines or infected vectors accidentally crushed along with food. Source: Pan-American Health Organization, 2009. Permissions: Reproduction is permitted with attribution.

In nature, the oral route is probably the oldest and most efficient route for parasite transmission and occurs mainly in 2 situations: 1) Ingestion of triatomine feces when the animal scratches the site of an insect bite with its mouth; or 2) predation of infected bugs or mammals. Other possible routes include fights between mammals (when the oral mucosa of a mammal comes in contact with the infected blood of the other mammal) and fomites contaminated by scent gland material of infected *Didelphis* sp. (Deane et al., 1984; PAHO, 2009), although these are still not empirically confirmed.

This biological plasticity of *Trypanosoma cruzi* results in a parasite widely dispersed in nature and immersed in transmission cycles that can be characterized as multivariable, complex, and peculiar to each locality (Jansen et al., 2015). Being found in the most diverse ecological niches from the southern parts of Argentina to southern United States, its transmission cycles are quite complex as it includes hundreds of mammalian species and vectors in scenarios of transmission that can be independent and overlapping with each other (Jansen et al., 2018).

As pointed out before, *Trypanosoma cruzi* has been circulating among wild mammalian fauna for at least 20 million years (depending on which hypothesis for the parasite's origin is considered). Humans are believed to have existed for no more than 500,000 years. Humans are estimated to have entered into the Americas (and therefore, exposed within this time to *T. cruzi* transmission cycles) only 15,000 to 24,000 years ago (Bourgeon et al., 2017). As such, there is evidence of human infections in mummies long before the arrival of Europeans in the Americas. Reports in paleoparasitological studies demonstrated the infection in 40% of the mummies examined in Chile, including at least one of them dating to 9,000 years. Almost half of the mummies had signs of megasyndromes (especially megacolon) and infections were seen among mummies from different cultural groups (Aufderheide et al., 2004). It is reported in many South American Aboriginal cultures the habit of feeding on raw or undercooked meat or even drinking the fresh blood of hunted mammals, which would result in infection by *T. cruzi* (Guhl et al., 2014). The contaminate vectorial transmission probably also occurred: 1) Inside caves used as human dwellings, due to the presence of kissing bugs associated with rocky environments, as in the case of *Triatoma brasiliensis* (Araújo et al., 2003); and 2) in peridomiciliary environments, as a consequence of the presence of small mammals such as guinea pigs that provided an abundant blood source for bugs in this environment (Aufderheide et al., 2004). These ancient scenarios demonstrate that there are no new or old ways of *T. cruzi* transmission. Humans have been exposed to the infection by both the contaminative and oral routes since they reached the Americas.

The species *Trypanosoma cruzi* is a monophyletic and extremely heterogeneous taxon that presents a multiclonal population structure influenced by mechanisms of gene exchange, epigenetic factors, introgression, and others (Stevens et al., 2001; Leonard et al., 2011). The heterogeneity of the parasite has been observed since its discovery, and thin and broad parasite forms observed in patients' blood were first associated with male and female gametocytes (Chagas, 1909). The first attempts to group *T. cruzi* isolates with similar characteristics dated from the 1970s: the biodemes, based on a distinct infection pattern in laboratory animals (Andrade et al., 1970) and the zymodemes, based on biochemical patterns of isoenzymes (Miles et al., 1977). The advent of molecular techniques and the proposal of distinct molecular targets in the 1980s and 1990s have revealed that this heterogeneity was much higher than previously thought. In the last decade, however, with the availability of gene sequence analysis in research labs, an even higher diversity of *T. cruzi* populations (and other related species) has emerged. In this scenario, the most employed gene targets are the small subunit (18S) ribosomal RNA gene and the nuclear glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) (Lima et al., 2015a; Lopes et al., 2018). These are the ones with the highest discriminatory power and those targets with the highest number of sequences deposited for comparison. Moreover, more easy accessibility to Next Generation Sequence (NGS) will probably uncover even higher trypanosomatid diversity, especially due the capacity to identify and characterize mixed infections directly on blood samples (Dario et al., 2017b; Pronovost et al., 2020).

Independent of the different molecular targets employed, 2 distinct and phylogenetically distant *Trypanosoma cruzi* populations have been recognized and were the basis for the first nomenclature consensus for *T. cruzi* (Luquetti et al., 1999). One year later, Brisse and colleagues (2000) proposed the grouping of *T. cruzi* isolates into 6 genotypes, or 6 **Discrete Typing Units** (**DTU**), named TcI to TcVI (Tc stands for *T. cruzi*). This is the basis of the current consensus of nomenclature: *T. cruzi* is a single species composed of 6 distinct DTUs (Zingales et al., 2009).

In addition to these DTUs, in 2009, a new *Trypanosoma cruzi* genotype, first associated with bats, was described: Tcbat (see Marcili et al., 2009). This genotype is phylogenetically close to TcI but represents a well separated clade. As a *T. cruzi* parasite, Tcbat is able to infect mice in experimental conditions. Although, its development in triatomines is more efficient in cave triatomines, which is the same pattern observed for another trypanosome associated with bats, *T. c. marinkellei* (See Marinkelle, 1982). Although first associated with bats, human infections by Tcbat have been reported in a Chilean mummy (Guhl et al., 2014) and a Colombian patient (Ramírez et al., 2014).

The currently most-accepted hypothesis to explain the establishment of the 6 DTUs points to TcI and TcII as the 2 ancestor lineages, and the emergence of the DTUs TcIII and TcIV as a result of a first hybridization process between them. A second and more recent hybridization process involving TcII and TcIII would have given rise to the current hybrid lineages TcV and TcVI (Westenberger et al., 2005).

The geographic distribution of *Trypanosoma cruzi* DTUs is not completely known and further knowledge of both the geographical and host-expanding data for each DTU will certainly result in distinct distribution maps (Jansen et al., 2015; 2018). However, some aspects can be highlighted: 1) TcI is the most dispersed DTU throughout the Americas; 2) outside South America, mainly TcI and TcIV are detected; 3) TcII occurs in the wild, but in restricted transmission foci and is the genotype associated with human infections in the former endemic areas of the disease (Jansen et al., 2015; 2018); 4) TcIII and TcIV are commonly found in wild mammals and the latter has been associated with oral outbreaks in the Amazon region (Santana et al., 2019); 5) TcV and TcVI are rarer than the others, both in humans and wild mammals; and 6) the subdividing genotypes of the *T. cruzi* population, previously known as zymodeme 2 (TCII in the first nomenclature consensus), are more recent. Because of that, ancient descriptions of TCI are still considered TcI, but TCII was subdivided into the other 5 subpopulations. This means that, except for TcI, all other *T. cruzi* DTUs are actually underestimated on the current distribution maps.

The widespread transmission of *Trypanosoma cruzi* to humans, which led to hundreds of thousands cases per year in the last century, was directly associated with the presence of the domiciliary bug *Triatoma infestans* (See Figure 6). This bug species originates from the Bolivian valleys of the eastern Andes, where it is found both in domiciles and in wild environments (Panzera et al., 2014). The domiciliary process of *Tri. infestans* occurred concurrently with the beginning of human settlements near forest environments. At the same time, the process of domestication of local guinea pigs near residences attracted the insects to the home environment (Aufderheide et al., 2004). After the colonization of the Americas by Europeans, the greater movement of people and materials favored the establishment of the intradomiciliary colonies of *Tri. infestans*, first in Bolivia and subsequently in other countries from South America, especially in those regions south of the Amazon basin. The presence of infected domiciliary bugs in stick houses was what Carlos Chagas found when he described the parasite and the associated vector (Chagas, 1909).

This classical scenario of transmission started to change in the 1970s, with insecticide campaigns to eliminate domiciliary colonies of this kissing bug species (Dias, 2007). In 1991, the governments of Argentina, Bolivia, Brazil, Chile, Paraguay, and Uruguay created the Southern Cone Initiative aiming to eliminate the intradomiciliary transmission of *Trypanosoma cruzi* by *Triatoma infestans,* which was achieved in 2006 (Dias, 2007). The certified interruption of this method of transmission, however, did not represent the end of contaminative vectorial transmission. This manner of transmission continues to occur, but now in the extradomiciliar environment, when humans expose themselves to wild transmission cycles, or in the intradomiciliar environment, usually associated with the invasion of wild vectors and not to domiciliary colonies (Dias et al., 2016). In both cases, control measures adopted against *Tri. infestans* are not effective. With the virtual elimination of *Tri. infestans* in some countries, including Brazil, the majority of the new reported cases are concentrated in the Amazon region, a previously considered non-endemic area due to the absence of *Tri. infestans* in the area. Only in Brazil, approximately 150–200 new cases of Chagas disease are reported per year (Dias et al., 2016). These cases are always associated with infected sylvatic kissing bugs that came in contact with humans.

These sylvatic bugs are immersed in a huge net of *Trypanosoma cruzi* transmission along with mammals from distinct orders. This transmission net resembles the trophic energy characteristic of food webs (Herrera et al., 2011). Both the oral route, through the predation of mammals and insects, and contaminative transmission are involved in the dispersion of this parasite in nature. Triatomines can be preyed upon by smaller mammals, and these by medium or top-chain carnivores (Herrera et al., 2011; Rocha et al., 2013). Moreover, the different mammal hosts are distributed in distinct forest strata and enable the parasite transmission in the most diverse habitats. This is especially important for mammals with generalist habits, like coatis, opossums, and felids, which frequent different forest strata and, thus, can connect parasite transmission cycles from different environments (Jansen et al., 2018).

Trypanosoma cruzi transmission can occur at all levels of the food web, and each group of mammals also presents varying importance as a reservoir of this parasite. In the pyramid base of consumers are the small terrestrial mammals. These mammals are excellent models of study because they comprise large populations and are usually the group of mammals with the highest biomass in any ecotope (Mills and Childs, 1998). In addition, they have a short lifespan and fast generational turnover, which allows the early identification of environmental changes. In the second level of the pyramid are the bats and medium-sized carnivores. They are classified as mesopredators, since they may be exposed to *T. cruzi* infection by predation of both infected vectors and small mammals. They are usually generalists and capable of exploiting

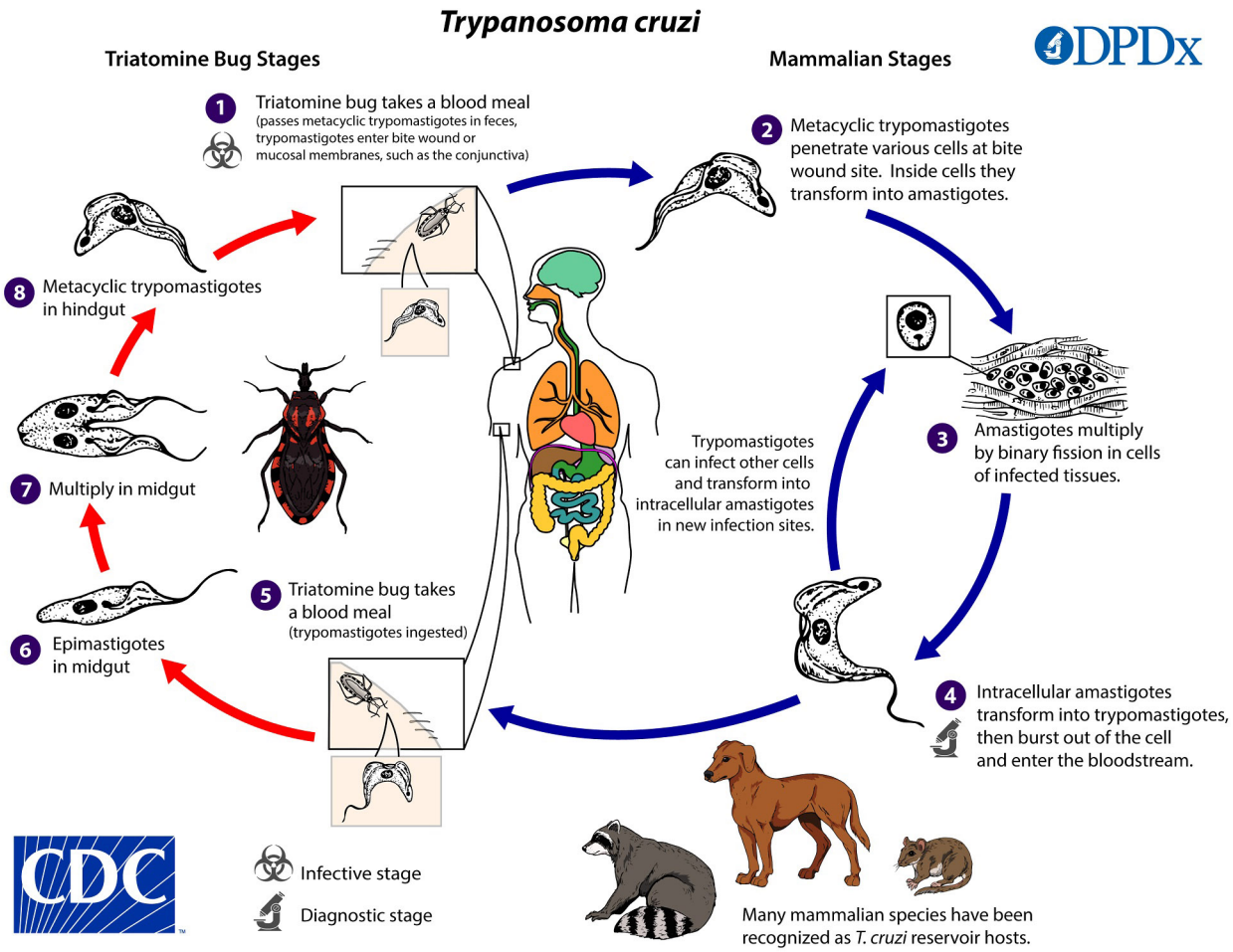

Figure 6. American Trypanosomiasis. *Trypanosoma cruzi*, is a parasitic protozoan that is the causative agent of Chagas disease (American trypanosomiasis). Currently, 6 distinct lineages of *T. cruzi* are classified into discrete typing units (TcI–VI), which vary in their geographic occurrence, host specificity, and pathogenicity. **Life cycle diagram:** An infected triatomine insect vector (or kissing bug) takes a blood meal and releases trypomastigotes in its feces near the site of the bite wound. Trypomastigotes enter the host through the bite wound or intact mucosal membranes, such as the conjunctiva (1). Inside the host, the trypomastigotes invade cells near the site of inoculation, where they differentiate into intracellular amastigotes (2). The amastigotes multiply by binary fission (3) and differentiate into trypomastigotes and then are released into the circulation as bloodstream trypomastigotes (4). Trypomastigotes infect cells from a variety of tissues and transform into intracellular amastigotes in new infection sites. Clinical manifestations can result from this infective cycle. The bloodstream trypomastigotes do not replicate (different from the African trypanosomes). Replication resumes only when the parasites enter another cell or are ingested by another vector. The kissing bug becomes infected by feeding on human or animal blood that contains circulating parasites (5). The ingested trypomastigotes transform into epimastigotes in the vector's midgut (6). The parasites multiply and differentiate in the midgut (7) and differentiate into infective metacyclic trypomastigotes in the hindgut (8). Other less common routes of transmission include blood transfusions, organ transplantation, transplacental transmission, and foodborne transmission (via food or drink contaminated with the vector and/or its feces). Source: United States Centers for Disease Control and Prevention, Global Health, Division of Parasitic Diseases and Malaria, 2021. Public domain.

distinct forest strata. At the top of the pyramid are those animals at the top of the food chain. They are mammals characterized by a wide geographic range and capacity for displacement, which are important conditions for parasite dispersion. In addition, they are considered bioaccumulators of orally transmitted parasites, as is the case for *T. cruzi* (see Herrera et al., 2011; Rocha et al., 2013).

Small mammals are excellent models of study for identifying spatial and temporal variation in *Trypanosoma cruzi* transmission. Concerning the spatial differences, the effect of habitat fragmentation on *T. cruzi* transmission among small mammals has been demonstrated by Vaz et al. (2007). Fragments of forest patches of different sizes (small, medium, and large) were investigated, in addition to a national park, as a

preserved control area. Vaz et al. (2007) observed that the greater the fragmentation of the wild environment, the lower the diversity of small placental mammals and the greater the abundance of marsupials. This different faunal composition was reflected in distinct infection prevalences, especially in the medium and large fragments (Vaz et al., 2007). Temporal differences were observed in the evaluation of infection prevalences in small mammals from the same locality (Jaguaruana, Ceará State, Brazil) during a 4-year follow-up. The anthropogenic devastation of the area resulted in lower species richness of placentals and a higher abundance of opossums. The consequence for *T. cruzi* transmission was an increase from an initial rate of 10% of the mammals infected to a rate higher than 50% after the fourth year (Jansen, unpublished data).

An example of a mesopredator that, throughout several years of study, demonstrated to be an important *Trypanosoma cruzi* reservoir is the coati (*Nasua nasua*) from the Pantanal region of Brazil. Distinct *T. cruzi* DTUs (TcI–TcIV) have been found in single mixed infections and long-term follow-up showed that coatis may present high and long-lasting parasitemias that are influenced by seasonality and gender (Herrera et al., 2008; 2011; Alves et al., 2016; Jansen et al., 2018). Coatis nest in very tall trees and their nests serve as microhabitats for several other mammals and insects, including kissing bugs that have been found to be infected by *T. cruzi* (see De Lima et al., 2015).

Golden lion tamarins—GLT (*Leonthopitecus rosalia*)—are also demonstrated to be competent *Trypanosoma cruzi* reservoirs (Lisboa et al., 2015). This callitrichid is an endangered species restricted to the Atlantic rainforest of Rio de Janeiro State, Brazil. It was during the GLT survey for *T. cruzi* that the presence of TcII (the DTU until then related only to human infections), was observed for the first time in the wild (Lisboa et al., 2000). An 11-year follow-up demonstrated that individuals of *L. rosalia* are able to maintain long-lasting infections and infectivity potential when infected by *T. cruzi* from both DTUs TcI and TcII (Lisboa et al., 2015).

Top-predator mammals are still poorly studied, mainly due to the difficulty in trapping and handling them, but are known as important bioaccumulators of *Trypanosoma cruzi*. In this sense, a positive correlation between the proportion of insects or other mammals in the diet and the infection rates by *T. cruzi* has been demonstrated (Rocha et al., 2013).

The composition of the local fauna may indicate which *Trypanosoma cruzi* DTUs will predominate in an area. *Didelphis* spp., for example, are known to maintain high and long-lasting parasitemias (as expressed by positive blood cultures), but only when infected by DTU TcI (Legey et al., 2003), while GLTs can maintain stable infections also by TcII (Lisboa et al., 2015). In contrast, wild and domestic canids usually exhibit a short period of high parasitemia, exactly as observed for humans (Jansen et al., 2018). This diversity explains the differences in the enzootic cycles of the parasite in different areas. In fact, each area is unique and has its own particularities (Jansen et al., 2015).

Currently, what will impact in the infection rates that have emerged in recent outbreaks of Chagas disease in the Americas include: 1) Control of domiciliary transmission of *Trypanosoma cruzi* by *Triatoma infestans*; 2) control of the parasite in the wild fauna of vectors and mammals; and 3) environmental disturbances that result in faunal modifications. With these in mind, and aiming to identify common risk factors that could be used in surveillance programs, domestic dogs were proposed as sentinels of imminent risk of transmission to humans (Roque and Jansen, 2008). In these outbreak areas, where a well-established wild transmission cycle of *Try. cruzi* was observed, dogs showed high prevalence of infection as diagnosed by serology, but did not contribute to the amplification of the parasite population, as attested by negative hemocultures (Roque et al., 2008; 2013). This proposal was further confirmed by spatial analysis using interpolation and map algebra tools, which also considered the environmental factors that interfered with the transmission. In this study, the authors confirmed the hypothesis that loss of wildlife richness was associated with higher parasitemias in wild fauna and higher serological prevalence in the associated canine population (Xavier et al., 2012).

In fact, the study of a multi-host zoonotic parasite such as *Trypanosoma cruzi* encourages analysis of the transmission to humans using a One Health approach (Thompson, 2013). Additionaly, implementation of the DAMA protocol is urgently needed in order to understand and take action in these and other parasites (Brooks et al., 2014; 2019). Parasite spillover or more properly known as ecological fitting (Janzen, 1985) is a phenomenon frequently amplified by changes in the landscape that: 1) Result in selection of individuals; 2) change the dynamics of transmission; and 3) in the case of zoonosis, will ultimately result in higher or lesser risk of emergence of human cases. Multidisciplinary studies are essential to better understand *T. cruzi* control and transmission, which are key conditions for successful surveillance programs. As a primarily enzootic parasite, *T. cruzi* transmission will never be eliminated, but prediction of new human cases and outbreaks is possible and urgently needed.

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Supplemental Reading

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12

Protozoa

Trypanosomatidae

Leishmania (Genus) and Leishmaniasis

Mary Ann McDowell and Jennifer Robichaud

Phylum Euglenozoa

Class Kinetoplastea

Order Trypanosomatida

Family Trypanosomatidae

Genus *Leishmania*

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Chapter 12

Leishmania (Genus) and Leishmaniasis

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Introduction

Leishmaniasis comprises a group of diseases caused by protozoans of the genus *Leishmania* (Ross, 1903b; Gibson, 1983) that are transmitted by the bites of phlebotomine sand flies. Of the 53 *Leishmania* species described, approximately 20 are known to be human pathogens (Table 1) (Akhoundi et al., 2016). The clinical manifestations of *Leishmania* infections range from lesions of the skin and mucous membranes to lethality (Herwaldt, 1999). Cutaneous leishmaniasis (CL), the most common form of the disease, comes in many forms including localized cutaneous leishmaniasis (LCL), characterized by a single, self-healing ulcer, diffuse cutaneous leishmaniasis (DCL) that presents as non-ulcerating lesions that are widespread on the body, disseminated cutaneous leishmaniasis (DCL), characterized by more than 10 lesions of mixed-types, mucocutaneous leishmaniasis (MCL), associated with destruction of the nasopharyngeal mucus membranes, visceral leishmaniasis (VL) where there is no initial cutaneous pathology and parasites spread to the visceral organs, and a complication of VL termed post kala-azar dermal leishmaniasis that is characterized by a erythmatous maculopapular rash that can extend to the entire body.

Leishmania species cause morbidity and mortality throughout large areas of the Old and New World and leishmaniasis is considered an emerging disease with an annual incidence of 0.9–1.7 million cases (Alvar et al., 2012). Leishmaniasis is found on all continents except Australia and Antarctica and is endemic in 98 countries, with 350 million people at risk of infection and causing 20,000 to 40,000 deaths per year (Alvar et al., 2012). An increased prevalence of *Leishmania*-HIV co-infection is responsible for the recent emergence of leishmaniasis in the Western world (Alvar et al., 2012; Desjeux and Alvar, 2003). Morbidity and mortality caused by leishmaniasis amount to an estimated 2.4 million disability-adjusted life-years (DALYs) (Desjeux, 2004) and the disease has recently been declared by the World Health Organization (WHO) as a category I Neglected Tropical Disease (NTD).

Historical Evidence

Evidence of *Leishmania*-like organisms from the blood of reptiles exists in fossil ambers of an extinct sand fly from Burma estimated to be approximately 100 million-years-old (Poinar et al., 2004a; 2004b) and from 20–30-million-year-old ambers from the Dominican Republic, although the vertebrate host is unknown in this case (Poinar, 2008). Human lesions, similar to those known as an ailment termed Oriental Sore, were first described in tablets from the Assyrian King Ashurbanipal in the 7th century BCE (= before current era), however, the information is thought to be derived from texts dating as old as 1500–2500 BCE (Steverding, 2017). In addition, Ancient Egyptian medical reports from 1500 BCE describe a condition known as Nile Pimple that is thought to refer to cutaneous leishmaniasis (Maspero, 1910). Physical evidence of *Le*. *donovani* DNA has been documented in Egyptian mummies dating as far back as 2050–1650 BCE (Zink et al., 2006) and immunological technique have been used to demonstrate *Leishmania* in a Peruvian mummy from 800 BCE (Frias et al., 2013).

In the Middle Ages, Arabic scientists made many references to descriptions of lesions, reminiscent of cutaneous leishmaniasis; the first being in 930 from the Baghdad region in Iraq (Edrissian et al., 2016) and a dermal condition known as Balkh Sore from Afghanistan by the Persian philosopher and physician Avicenna (980–1037) (Severding, 2017). In the New World, disfiguring facial lesions are depicted on pre-Columbian ceramics from the 5th century (Tuon et al., 2008) and skulls dating back to the 11th century discovered in northern Chile have morphological and molecular evidence of leishmaniasis in the New World (Costa et al., 2009).

Table 1. Clinical and Epidemiological Characteristics of *Leishmania* Species

***Leishmania* species**	**Subgenus**	**Old World and/or New World**	**Proven vector species**	**Clinical manifestation**	**Primary reservoir hosts**	**Distribution**	**Estimated global incidence**
Le. donovani	*Leishmania*	OW	*P. alexandri* *P. argentipes* *P. martini* *P. orientalis*	VL, PKDL	Dogs, foxes, opossums, rodents	Central Africa, South Asia, Middle East, India, China	50,000–90,000 VL cases; unknown number of PKDL cases
Le. tropica	*Leishmania*	OW	*P. arabicus* *P. guggisbergi* *P. rossi* *P. saevus* *P. sergenti*	LCL, RCL, rarely VL	Rock hyraxes	Central Africa, North Africa, Middle East, Central Asia, India	200,000–400,000 CL; unknown number of viscerotropic or RCL
Le. aethiopica	*Leishmania*	OW	*P. longipes* *P. pedifer* *P. sergenti*	LCL, DCL, DsCL, MCL	Rock hyraxes	East Africa	20,000–40,000 CL; breakdown of LCL, DCL, DsCL, MCL unknown
Le. major	*Leishmania*	OW	*P. duboscqui* *P. papatas,* *P. salehi*	CL	Gerbils, other rodents	Central Africa, North Africa, Middle East, Central Asia	230,000–420,000 LCL
Le. infantum	*Leishmania*	OW/NW	*Lu. almerio,* *Lu. cruzi* *Lu. evansi* *Lu. longipalpis* *Lu. migonei* *P. ariasi* *P. balcanicus* *P. brevis* *P. chineesis* *P. kandelakii* *P. langeroni* *P. longiductus* *P. perfillewi* s.l. *P. perniciosus* *P. sichuanensis* *P. smimovi* *P. tobbi* *P. turanicus* *P. wui*	LCL, VL	Dogs	North Africa, Mediterranean basin, Middle East, Central Asia, North America, Central America, South America	6,200–12,000 VL in Old World; 4,500–6,800 VL in New World; Unknown number of CL cases
Le. mexicana	*Leishmania*	NW	*Lu. ayacuchensis* *Lu. olmeca olmeca,* *Lu. ovallesi* *Lu. anthaphora*	LCL, DCL, DsCL	Forest rodents	North America (including the United States), Central America, South America	†Included in the 187,200–300,000 total cases of New World CL
Le. amazonensis	*Leishmania*	NW	*Lu. faviscutellata* *Lu. longipalpis* *Lu. nuneztovari anglesi* *Lu. omeca novice* *Lu. olmeca reducta*	LCL, DCL, DsCL	Rain forest rodents, marsupials, foxes, bats	South America	†Included in the 187,200–300,000 total cases of New World CL
Le. venezuelensis	*Leishmania*	NW	*Lutzomyia* spp. implicated but not proven		Unknown	Northern South America	†Included in the 187,200–300,000 total cases of New World CL

Table 1. Clinical and Epidemiological Characteristics of *Leishmania* Species (continued)

***Leishmania* species**	**Subgenus**	**Old World and/or New World**	**Proven vector species**	**Clinical manifestation**	**Primary reservoir hosts**	**Distribution**	**Estimated global incidence**
Le. braziliensis	*Viannia*	NW	*Lu. carrerai* *Lu. complexa* *Lu. fischeri*	LCL, MCL, DCL, RCL	Opossums, rain forest rodents	Western Amazon basin, South America, Central America	†Included in the 187,200–300,000 total cases of New World CL
Le. guyanensis	*Viannia*	NW	*Lu. anduzei* *Lu. ayacuchensis* *Lu. shawi* *Lu. umbratilis,* *Lu. whitmani*	LCL, DsCl, MCL	Sloths	Northern South America	†Included in the 187,200–300,000 total cases of New World CL
Le. lainsoni	*Viannia*	NW	*Lu. nuneztovari anglesi* *Lu. ubiquitalis*	LCL	Forest rodents	Brazil, Bolivia, Peru	†Included in the 187,200–300,000 total cases of New World CL
Le. lindenbergi	*Viannia*	NW	*Lu. atunesi* implicated	LCL		Brazil	†Included in the 187,200–300,000 total cases of New World CL
Le. naiffi	*Viannia*	NW	*Lu. ayrozai* *Lu. squamiventris*	LCL	Armadillos, rodents	Brazil, French Guiana	†Included in the 187,200–300,000 total cases of New World CL
Le. panamensis	*Viannia*	NW	*Lu. gomezi* *Lu. harmanni* *Lu. panamensis* *Lu. trapidol* *Lu. yulli*	LCL, MCL	Sloths	Central America, South America	†Included in the 187,200–300,000 total cases of New World CL
Le. peruviana	*Viannia*	NW	*Lu. ayacuchensis* *Lu. peruensis*	LCL, MCL	Opossums, dogs	Peru, Bolivia	†Included in the 187,200–300,000 total cases of New World CL
Le. shawi	*Viannia*	NW	*Lu. whitmani*	LCL	Sloths, rodents	Brazil	†Included in the 187,200–300,000 total cases of New World CL
Le. martiniquensis	*Mundinia*	OW/NW	Unknown	LCL, VL	Horses, cattle	Martinique, Thailand, Central Europe, United States	Unknown
Le. orientalis	*Mundinia*	OW	Unknown	LCL	Unknown	Thailand	Unknown
Le. colombiensis	*Mundinia*	NW	*Lu. hartmanni*	LCL, VL	Sloths	Colombia	†Included in the 187,200–300,000 total cases of New World CL

VL = visceral leishmaniasis, PKDL = post kala-azar dermal leishmaniasis, DCL = diffuse cutaneous leishmaniasis, DsCL = disseminated cutaneous leishmanniasis, LCL = localized cutaneous leishmaniasis, MCL = mucocutaneous leishmaniasis, RCL = recidivans cutaneous leishmaniasis, *P.* = *Phlebotomus*, *Lu.* = *Lutzomyia*. † Accounting of CL cases in the New World is complex as there are multiple *Leishmania* species circulating in the same geographical area, variable clinical manifestations associated with each species and species identification is rarely reported. Table compiled from multiple authoritative sources.

In modern times (16th–19th century) there are many reports of cutaneous leishmaniasis (CL), generally conditions named for the location they were acquired (for example, Aleppo boil, Baghdad boil, Jericho boil); interestingly, many of these terms are still used today (Severding, 2017). The earliest report of a disease likely to be visceral leishmaniasis did not occur until the 19th century with a description of an outbreak of a disease in 1824–1827 that caused emaciation, enlarged spleens, acute anemia, intermittent fever, and a dried, scaly appearance of the skin (Gibson, 1983; Twining, 1827). The Hindi term kala-azar that roughly translates to black fever and referring to the grayish discoloration of the skin was coined late in the 19th century to describe VL (Steverding, 2014). In the 20th century the Scottish pathologist William Boog Leishmana discovered ovoid bodies from the spleen of a soldier who died from a disease of emaciation while serving at Dum Dum, a town in India, and termed the disease Dum Dum Fever (Leishman, 1903); at the same time Charles Donovan identified similar bodies from splenic aspirates of Indian patients (Donovan, 1903), but the 2 scientists could not agree if the parasites were trypanosomes or a new species. Ronald Ross, investigating kala-azar in India, found similar parasites from spleens of patients with chronic splenomegaly (Ross, 1903a) and settled the controversy, declaring the parasites a new species named *Leishmania donovani* (Ross, 1903b). Leishmaniasis was not reported in the new world until the 20th century; CL reported from Brazil in 1909, referred to as Baurú ulcers (Carini and Paranhos, 1909; Lindenberg, 1909), and VL, also from Brazil, in the 1930s (da Cunha et al., 1937).

Leishmaniasis continues to be a major global health threat and, although endemic in Europe, Africa, Asia, and America, 90% of cases occur in just 13 countries (Afghanistan, Algeria, Bangladesh, Bolivia, Brazil, Columbia, Ethiopia, India, Iran, Peru, South Sudan, Sudan, and Syria). The burden of leishmaniasis is largely underestimated, however, due to misdiagnosis and lacking surveillance systems. Human migration, political instability, climate change, and warfare is expanding *Leishmania*-endemic regions and increasing the propensity for epidemics worldwide. As an example, cutaneous leishmaniasis is currently spreading as refugees move from Syria through Turkey into Europe and other regions throughout the world (Hayani et al., 2015; Nimer, 2018). As a consequence, there is a significant risk that cutaneous leishmaniasis (CL) will reemerge in southern Europe, where the natural sand fly vectors for *Leishmania tropica* and *Le. major* are already endemic, and travelers, not only refugees, may be affected (Di Muccio et al., 2015).

Nomenclature and Morphology

Leishmania species are flagellated, single cell, protozoans in the order Kinetoplastida and the family Trypanosomatidae. As with other members of this group, *Leishmania* spp. are characterized by a unique mitochondrian that contains a kinetoplast at the base of the flagellum. The kinetoplast contains DNA (kDNA) that represents 10–20% of the total cellular DNA (Simpson, 1987) and is organized as an interlocked network containing dozens of maxi- and thousands of mini-circles. Mini-circles encode for guide RNAs (gRNAs) that function in a unique RNA editing mechanism (Read et al., 2016) and maxi-circles are analogous to mitochondrial DNA in other eukaryotes. Due to the high copy number of mini-circles, kDNA can be utilized for diagnostic purposes via polymerase chain reaction (PCR) (Van der Auwera and Dujardin, 2015; Galluzzi et al., 2018).

Historically, *Leishmania* species were classified based on the clinical symptoms they generated, those causing CL considered as *Le. tropica* and those causing VL as *Le. donovani*. As more parasites from around the world were examined and molecular techniques became available, it became clear that there were many different subgroups within the genus *Leishmania* (Lainson and Shaw, 1987). Today, at least 4 subgenera exist for the genus *Leishmania*: *Sauroleishmania, Leishmania, Mundinia,* and *Viannia* (Espinosa et al., 2018), the latter 3 subgenera contain species known to infect humans. Species of the subgenera *Sauroleishmania* infect lizards do not cause human disease. The vast majority of species that cause human disease belong to the *Leishmania* and *Viannia* subgenera; those belonging to *Leishmania* are found in both the Old and New World and those belonging to *Viannia* are exclusively found in the neotropics. These 2 subgenera can be distinguished by the location the parasites grow within the sand fly gut, *Leishmania* (*Leishmania*) spp. found anterior to the pylorus and *Leishmania* (*Viannia*) in the mid and hindgut.

Life Cycle

Leishmania are digenic parasites, completing their life cycle within 2 hosts. These parasites develop within the alimentary track of phlebotomine sand flies (order Diptera: family Psychodidae, subfamily Phlebotominae) and are transmitted to humans when female sand flies blood feed (Figure 1). Once deposited in the vertebrate host the parasites are quickly phagocytosed by cells of the mononuclear phagocyte system, where they establish their niche. *Leishmania* have 2 primary morphological forms, long (5–15 μm), extracellular promastigotes with long flagella in sand flies and small (3–5 μm), intracellular amastigotes with rudimentary flagella within vertebrate hosts.

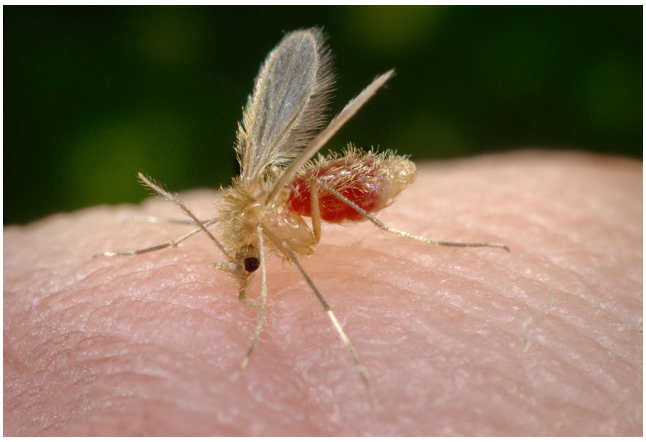

Figure 1. A *Phlebotomus papatasi* sand fly, which landed atop the skin surface of the photographer who had volunteered himself as host for this specimen's blood meal. The sand flies are members of the Dipteran family Psychodidae and the subfamily Phlebotominae. This specimen was still in the process of ingesting its blood meal, which is visible through its distended transparent abdomen. Source: United States Public Health Image Library, image 10275; J. Gathany, 2006. Informed consent granted by human subject: J. Gathany, 2006. Public domain.

Leishmania are obligate intracellular parasites that survive and multiply within the mature phagolysosome compartment of mononuclear phagocytes. Metacyclic promastigotes that enter the skin are quickly phagocytosed either directly by macrophages or dendritic cells or indirectly through apoptotic infected neutrophils that are rapidly recruited to the bite site (van Zandbergen et al., 2004). *Leishmania* do not actively invade host cells, but rely on the phagocytic capacity of these cells to gain entry. This process is receptor-mediated (Alexander and Russell, 1992; Chang and Dwyer, 1978), eventually resulting in the formation of a phagolysosome, and is known to induce host-cell signal transduction pathways (Guy and Belosevic, 1993). Many receptors are known to mediate entry of *Leishmania* parasites including, complement receptors 1 and 3, FcReceptors, mannose receptors, scavenger receptors, and fibronectin receptors (Ueno and Wilson, 2012). Recently, Toll-like receptors also have been implicated during *Leishmania* infection (Chauhan et al., 2017). These receptors are part of the innate system of pattern recognition, a system used by host cells to discriminate between infectious non-self and self. Upon interaction with ligand, these receptors initiate signal transduction pathways that ultimately lead to the modulation of phagocyte functions. The repetitive structure and glycan modifications associated with many *Leishmania* cell surface molecules serve as pathogen associated molecular patterns (PAMPs) that are recognized by the pattern recognition receptors (PRRs) (Podinovskaia and Descoteaux, 2015).

Once phagocytosed, *Leishmania* parasites are delivered to an intracellular vacuole termed a phagosome. Phagocytosis of a foreign body typically results in phagosome fusion with lysosomes allowing for degradation of phagosomal contents within 30 minutes. This *Leishmania* delays the phagosome maturation process, taking approximately 5 hours, the delay theoretically allowing enough time for the parasites to start differentiating toward the more resistant amastigote stage (Desjardins and Descoteaux, 1997; Scianimanico et al., 1999); full differentiation into amastigotes occurs between 24 and 48 hours post engulfment. *Leishmania* amastigotes multiply within the phagolysosome and eventually escape from the cell by a poorly defined mechanism and re-invade other phagocytes. Different species of *Leishmania* induce morphologically distinct phagolysosomes. *Leishmania*. *mexicana* resides in spacious vacuoles that contain many amastigotes, whereas *Le*. *major* and *Le*. *donovani* induce tight fitting phagosomes that contain only 1 amastigote (McConville et al., 2007).

When a sand fly ingests blood, it is retained within a peritrophic matrix in the abdominal midgut until digestion is completed. *Leishmania* amastigotes within infective blood transform into procyclic promastigotes within 12–18 hours within the peritrophic matrix. Procyclic promastigotes rapidly divide within the digesting blood meal and differentiate into longer, more motile nectomonads. When blood digestion is completed and excreted (~ 3–5 days), nectomonads are found in the gut attached to the epithelial cell microvilli via their flagella (Sacks and Kamhawi, 2001). Approximately 7 to 10 days following the initial blood meal the parasites transform into short, actively dividing forms called leptomonads and migrate to the thoracic midgut and stomodeal valve (an invagination of the foregut into the midgut). Leptomonads produce a substance, promastigote secretory gel that imbeds the parasites (Gossage et al., 2003). The end-point of parasite development in the sand fly is differentiation into the infectious stage, metacyclic promastigotes. These infectious forms are short, slender forms with a flagella twice as long as the body and are highly motile (Sacks and Perkins, 1984). Different than *Plasmodium* sporozoites that reside in mosquito salivary glands and are injected during blood-feeding, *Leishmania* metacyclic promastigotes do not invade the salivary glands and are regurgitated. The stomodeal valve is physically obstructed by the promastigote secretory gel, interfering with blood-feeding and leading to regurgitation and persistent but intermittent feeding (Rogers et al., 2004).

Clinical Manifestations

Leishmaniasis is a group of diseases characterized by a range of clinical symptoms that fall under 2 primary

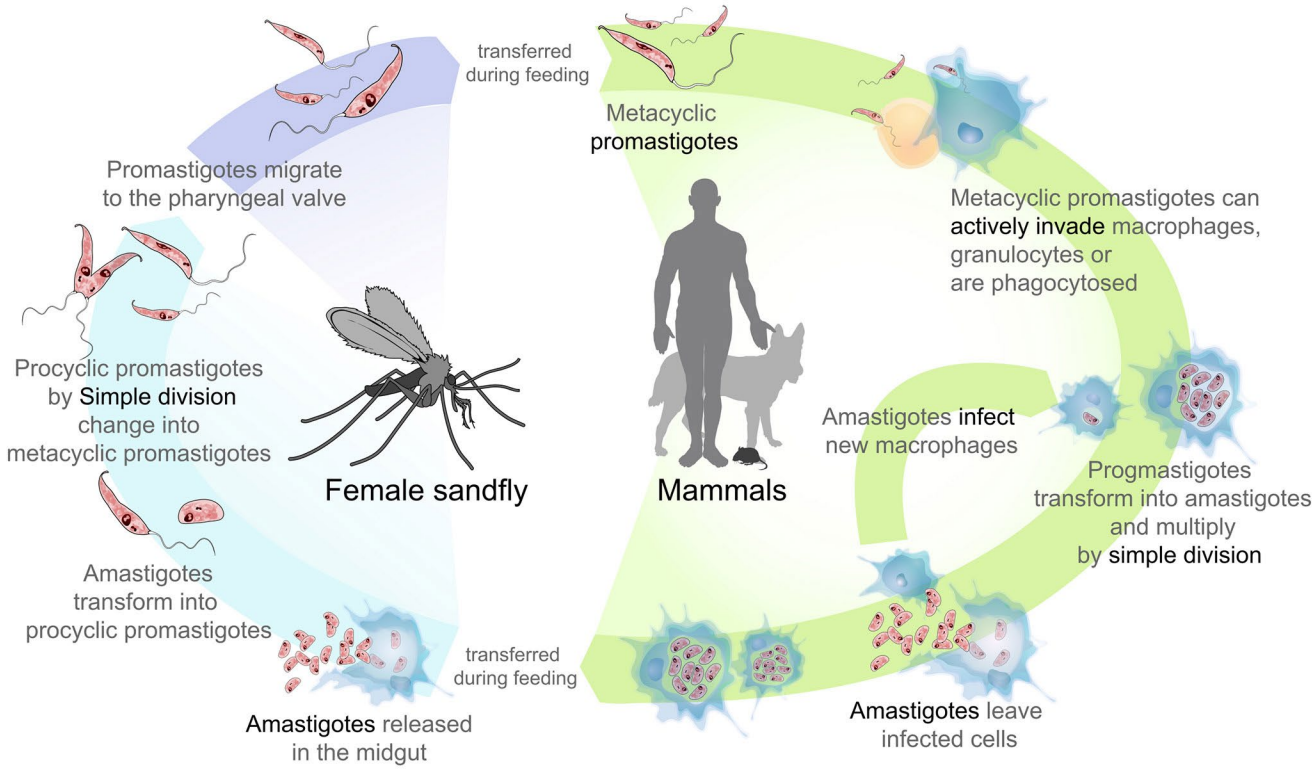

Figure 2. Life cycle of the parasites from the genus *Leishmania*. Source: Wikimedia Commons; Ruiz Villarreal, 2008. Public domain.

categories, **cutaneous leishmaniasis** (CL) and **visceral leishmaniasis** (VL). Although this is an ever-changing number as new species are described and taxonomic phylogenies revised, there currently are 22 *Leishmania* species known to be pathogenic to humans (Table 1; PAHO, 2024). The different pathological manifestations that present are primarily associated with the *Leishmania* spp. initiating the infection.

Cutaneous Leishmaniasis (CL)

The World Health Organization (WHO) estimates that 1.2 million annual cases of CL worldwide with 70–75% of the cases occurring in just 10 countries, Afghanistan, Algeria, Brazil, Colombia, Costa Rica, Ethiopia, Iran, North Sudan, Peru, and Syria. However, these statistics are largely underestimated due to misdiagnosis and lack of surveillance systems (see Figures 3 and 4).

There has been a recent increase in the incidence of CL in the United States due to several factors, including travel to endemic regions, immigration, and military operations. Twenty cases of CL were identified in United States military personnel deployed during the 1990–1991 Gulf War in the Middle East. More recently, CL has had a profound effect on United States troops; 1,287 deployed United States military personnel contracted leishmaniasis during campaigns in Iraq and Afghanistan during 2001–2006 and 522 cases in personnel who served in southwest and central Asia (Pavli and Maltezou, 2010). In addition, CL is found in the United States, being endemic in southern Texas and may be spreading north to Oklahoma (Clarke et al., 2013; Kipp and Hergert, 2019).

The most common form of this CL is **localized cutaneous leishmaniasis (LCL)**, characterized by localized, self-limiting cutaneous ulcers and powerful lifelong immunity upon healing. The lesions most often occur on exposed areas of skin where the sand fly vector can take a blood meal and begin as papules that eventually ulcerate. The patients are generally well and slight pain may or may not be associated with the lesions. Multiple lesions of this condition usually correspond to multiple sand fly bites. Although the lesions eventually heal (3–18 months), even without treatment, they are associated with substantial scarring and often social stigm (Bennis et al., 2018). Ancient civilizations noted that individuals who had healed from Oriental sores were protected from further disease (Steverding, 2017). Leishmanization, the practice of innoculating individuals with exudates from active lesions into the buttocks of young children, particularly girls, has been used in the Middle East and Central Asia for centuries to prevent the development of facial scars

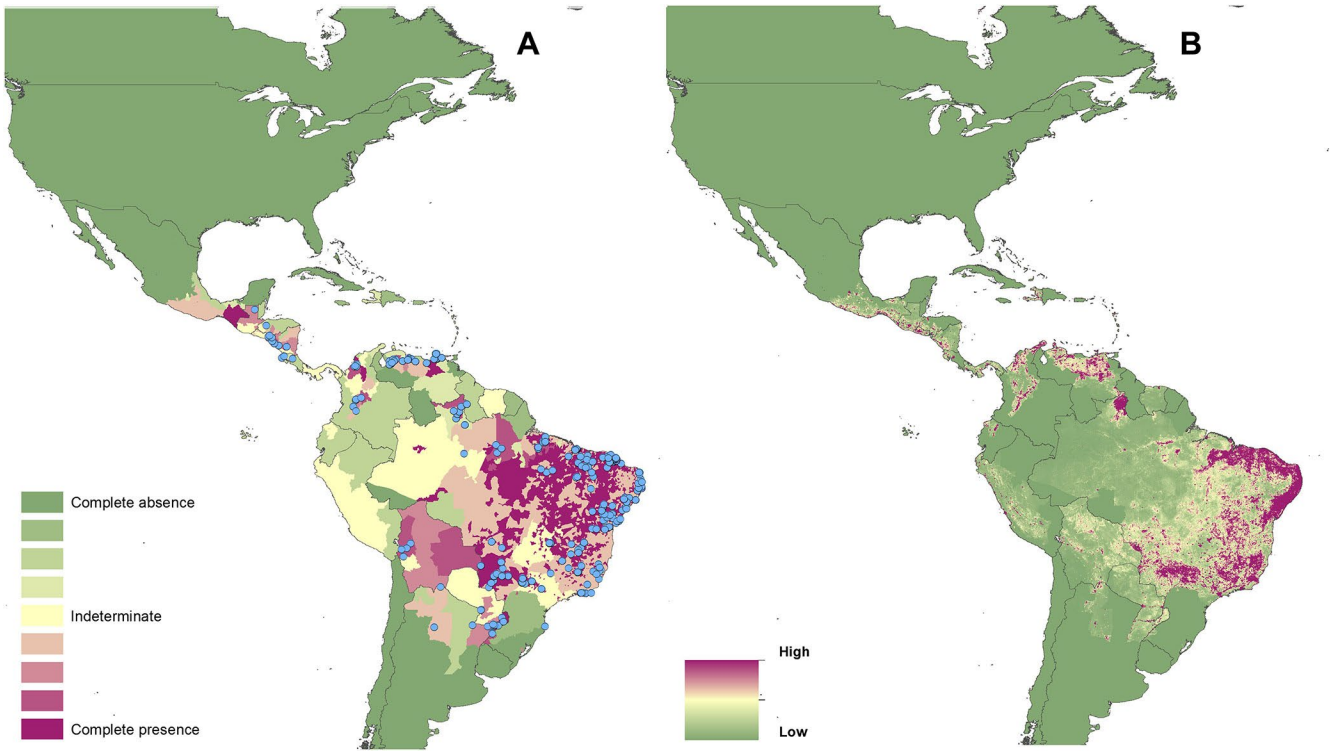

Figure 3. Reported and predicted distribution of cutaneous leishmaniasis in the New World. A) Evidence consensus for presence of the disease ranging from green (complete consensus on the absence: −100%) to purple (complete consensus on the presence of disease: +100%). The blue spots indicate occurrence points or centroids of occurrences within small polygons. B) Predicted risk of cutaneous leishmaniasis from green (low probability of presence) to purple (high probability of presence). Source: Pigott et al., 2014. License: CC0 1.0 Universal.

(Steverding, 2017). Today, leishmanization is still the only effective leishmaniasis vaccine that leads to lifelong protection (Nagill and Kaur, 2011).

Diffuse cutaneous leishmaniasis (DCL) is less common and characterized by multiple slow growing, non-tender nodules that disseminate all over the body. The nodules do not ulcerate and typically contain large numbers of parasites. DCL is a chronic disease that can persist for 20 years or more (Hashiguchi et al., 2016) and is often misdiagnosed as lepromatous leprosy. The condition is thought to reflect an underlying lack of a cellular immune response as evidenced by poor ability to respond to *Leishmania* antigen (Scott and Novais, 2016).

Disseminated cutaneous leishmaniasis (DsCL) is a non-chronic condition where there are multiple (≥ 10) lesions of different types, often ulcerating on more than 2 parts of the body. Patients are strongly positive for the Leishmanin skin test, indicating strong cellular immunity (Hashiguchi et al., 2016).

Recidivans cutaneous leishmaniasis (RCL) is a reactivation after a lesion is healed, usually within 2 years. Reactivated lesions typically encircle the previous scar and can be difficult to treat (Gitari et al., 2018). This condition is primarily associated with *Leishmania tropica* infection.

Mucocutaneous leishmaniasis (MCL) occurs after an initial local cutaneous lesion has healed. The parasites disseminate to the nasopharyngeal mucus membranes. The disease is characterized by destruction of the nasal septum, lips, palate, and sometimes larynx. Patients with MCL are at risk of death due to aspiration pneumonia.

Leishmania major.

Cutaneous leishmaniasis in the Old World accounts for the majority of global CL incidence in the world. The majority of these cases are caused by either *Leishmania major* or *Le. tropica* (Table 1). *Leishmania major* is endemic in the Middle East and North, West, and East Africa, and Central Asia. Confirmed vector species include *Phlebotomus duboscqi* in western and eastern Africa, *P. papatasi* and *P. bergeroti* in the Middle East, North Africa, and Europe, and *P. salehi* in India, Iran, and Pakistan. *Leishmania major* is a zoonosis with several rodent species implicated as reservoirs, differing geographically.

Leishmania major infection predominately manifests as LCL with lesions starting as a small papule, occasionally with nodules, at the site of the sand fly bite. Lesions generally

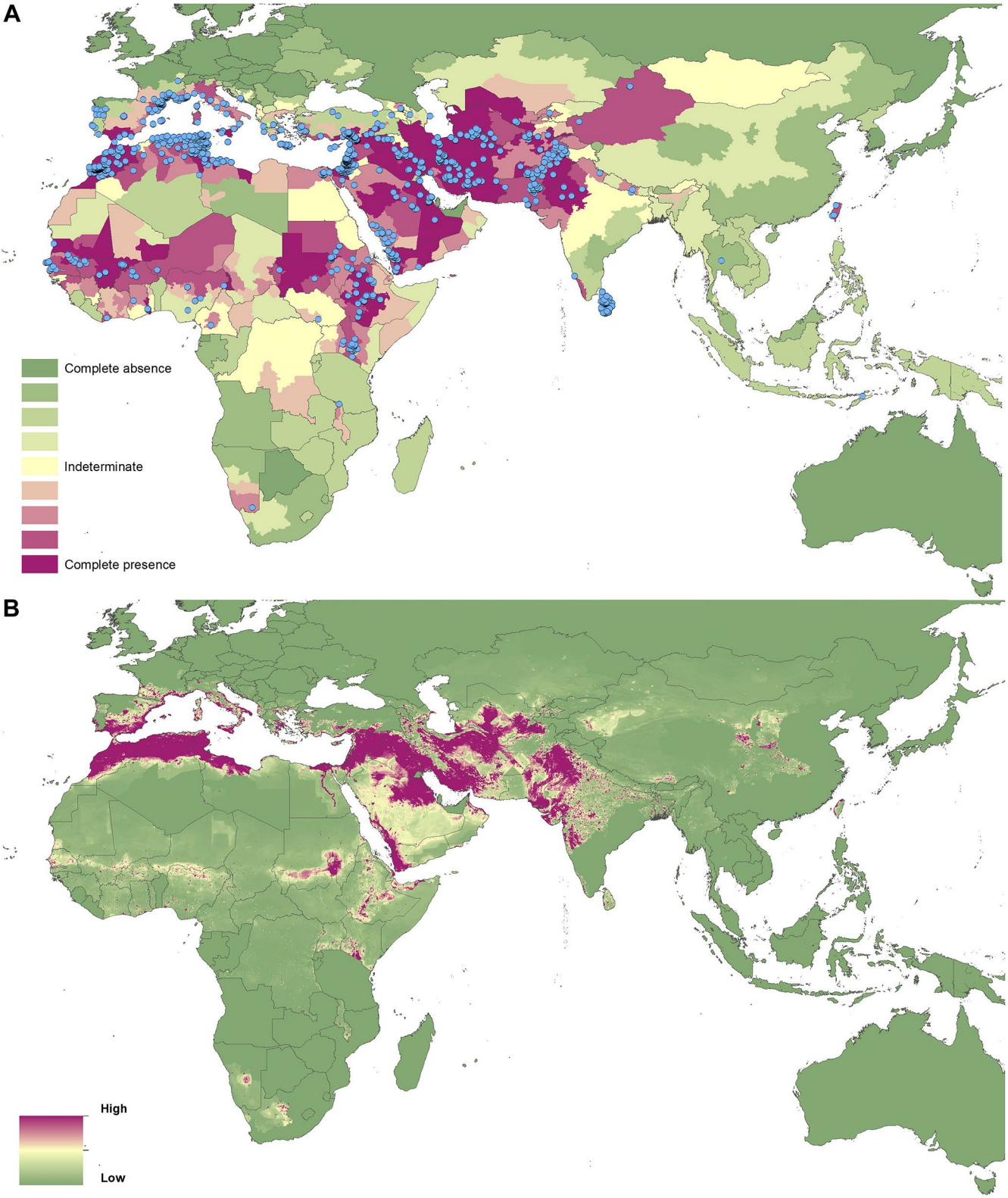

Figure 4. Reported and predicted distribution of cutaneous leishmaniasis in the Old World. A) Evidence consensus for presence of the disease ranging from green (complete consensus on the absence: −100%) to purple (complete consensus on the presence of disease: +100%). The blue spots indicate occurrence points or centroids of occurrences within small polygons. B) Predicted risk of cutaneous leishmaniasis from green (low probability of presence) to purple (high probability of presence). Source: Pigott et al., 2014. License: CC0 1.0 Universal.

occur on the head, neck, or extremities where sand flies have access to the skin. The incubation period is generally 2–4 weeks but may range from days to years (Goto and Lindoso, 2010). The papules enlarge and develop into large painless ulcers with a raised darker pigmented border (Darmstadt et al., 1993; Morris-Jones and Weber, 2004). The lesions are typically wet and associated with severe inflammation (Burza et al., 2018). Rarely is there more than 1 lesion present and self-healing usually occurs within 1 year (Melby et al., 1992); however, there is severe scarring due to the necrosis and inflammation associated with the lesions (Burza et al., 2018). Those that heal become immune to further infection; this natural occurring immunity provides the rational for vaccine development.

Leishmania tropica.

The geographic range of *Leishmania tropica* includes Central and North Africa, the Middle East, and Central Asia. *Le. tropica* infection has generally thought of as an anthroponotic infection, however, in some locations it appears to be a zoonosis (Kamhawi et al., 1995; Saliba et al., 1997; Talmi-Frank et al., 2010), with the rock hyrax as a reservoir host (Talmi-Frank et al., 2010). Vector species include *Phlebotomus guggisbergi*, *P. rossi*, *P. saevus*, and *P. arabicus*, and *P. sergenti*, with the latter 2 being the most common.

Similar to *Leishmania major* infections, the majority (76%) of lesions occur on the head and neck, followed by 30–36% on the extremities and trunk (Solomon et al., 2014). Most patients present with 1 LCL lesion and 95% have fewer than 3 lesions (Solomon et al, 2014). Lesions due to *Le. tropica* infection typically take longer to heal than those caused by *Le. major*, with the majority healing within 2 years (Handler et al., 2015). The lesions ulcerate but are dry in nature (Burza et al., 2018). In some cases, lesions can develop into hyperkeratotic plaques that resemble large warts (Burza et al., 2018). Individuals that have healed from a previous cutaneous ulceration due to *Le. tropica* can relapse, causing RCL (Burza et al., 2018). This chronic relapsing form of the disease generally begins with nodules, with lesions forming at the periphery of the old lesion scar.

Of the 32 cases of leishmaniasis identified in United States soldiers during the 1990–1991 Desert Storm Campaign, 12 were characterized as viscerotropic caused by *Leishmania tropica* (Hyams et al, 1995). Viscerotropic leishmaniasis is a syndrome where parasites spread to visceral organs and is associated with a prolonged systemic illness that includes fever, malaise, abdominal pain, and intermittent diarrhea but does not progress to fatal VL (Magill et al., 1993).

Most recently, the civil war in Syria has been associated with a large epidemic of *Leishmania tropica* CL (Rehman et al., 2018). Before the onset of the civil war, WHO statistics indicate approximately 14,000 new CL cases per year in Syria with incidence increasing to 27,825 in 2010 (Rehman et al. 2018) and to 89,357 in 2019 (WHO, 2024). The governmental surveillance system for leishmaniasis has lost access to some provinces in Syria so only sparse reliable data have been published since 2011. A recent humanitarian organization reported nearly 65,000 cases from a few provinces in northern Syria, with the majority associated with *Le. tropica* infection, although *Le. major, Le. infantum,* and *Le. donovani* are also present (Rehman et al, 2018). Syrian refugees are migrating to nearby areas where suitable vectors are endemic, expanding the epidemic.

Leishmania aethiopica.

Leishmania aethiopica is a zoonotic disease, primarily occurring in the highland areas of Ethiopia with the rock hyrax as a reservoir. The annual burden of CL in Ethiopia is approximately 20,000–40,000 cases per year (Alvar et al., 2012) with 99% with *Le. aethiopica* as the etiological agent (van Griensven et al., 2016). While LCL is the most frequent manifestation, clinical symptoms are diverse with MCL and DCL also being relatively common (Padovese et al., 2009). The reasons for the diverse symptomology are unclear and may involve many factors, including the high level of genetic polymorphism exhibited by this species (Pratlong et al., 2009).

Leishmania aethiopica LCL lesions are slower to develop, typically do not ulcerate, and are more chronic compared to other LCL lesions, requiring 2–5 years to heal (Handler et al., 2015). Both MCL and DCL caused by *Le. aethiopica* are reportedly less responsive to common antileishmanial drugs (Padovese et al., 2009). Treatment for DCL has been notoriously difficult and even if lesions regress, relapse is common upon cessation of chemotherapy (van Griensven et al., 2016).

Leishmania mexicana.

Cutaneous leishmaniasis epidemiology in the Americas is extremely complex with multiple *Leishmania* species with overlapping geographical distributions, a variety of sand fly vectors, and many different reservoir hosts. Due to this complexity and that reporting structures do not require species identification, the exact incident levels of each species is difficult to discern. *Leishmania mexicana* is endemic in North America, Central America, and South America and is the species endemic to the United States.

Forest rodents serve as reservoir hosts in most of the Americas, with 3 species of woodrats serving as reservoir hosts in the United States, including *Neotoma micropus, N. albigula,* and *N. floridana*. Several confirmed vectors have

been identified (Table 1); *Lutzomyia anthaphora* is the only confirmed vector in the United States (Endris et al., 1987; McHugh et al., 1993).

Leishmania mexicana lesions resemble those caused by *Le. major* being generally less severe and healing quickly. However, they can be slow to develop, sometimes taking up to 6 months, and can persist for 20 years (Handler et al., 2015). Lesions occur roughly 50% of the time on the ear, a manifestation referred to as chiclero's ulcer; the term is used because LCL on the ear is common among men that visit the forests to collect chicle (natural form of gum). DCL rarely presents with infection with *Le. mexicana*.

Leishmania braziliensis.

Leishmania braziliensis, endemic in South America and Central America (Grimaldi et al., 1987) is known in some places locally as espundia. Infection with *Le. braziliensis* results in severe cutaneous lesions and is associated with satellite subcutaneous nodules and lymph node involvement (Melby et al. 1992). LCL lesions caused by *Le. braziliensis* generally ulcerate and may heal within 6 months; however, 2–5% of cases develop into MCL and these require treatment (Burza et al., 2018). Although several species have been implicated in MCL, *Le. braziliensis* accounts for the majority of cases in the New World (Strazzulla et al., 2013).

MCL usually presents after the healing of a primary skin lesion but can begin to develop prior to lesion resolution (Daneshbod et al., 2011). Mucosal involvement normally appears within 2 years of LCL but has been reported to take up to 30 years (Samady et al., 1996). Although general subsequent mucosal involvement generally occurs in < 5% of cases, it may be as high as 20% in certain regions (David et al., 1993).

It is unknown why some patients are more susceptible to MCL. *Leishmania* RNA virus (LRV)-1 was identified in both *Le. braziliensis* and *Le. guyanensis*, both associated with MCL, leading to the hypothesis that MCL is actually virally mediated (Scheffter et al., 1995). It is hypothesized that virally infected *Leishmania* are recognized by host PRR that induce killing of the parasite and allowing dispersal of the virus. This dispersal in turn triggers a metastatic hyperinflammatory reaction, resulting in tissue damage (Weinkopff et al., 2013; Zangger et al., 2013).

Additional New World *Leishmania* Species.

Leishmania amazonensis is restricted to South America and is associated with LCL, DCL, and DsCL. *Leishmania peruviana* causes LCL, a disease known as uta in preschool age children in the Peruvian Andes (Davies et al., 1997). Ulcera de Bejuco is CL caused by *Le. panamensis*, characterized by shallow ulcers that metastasize along lymphatic vessels. There is no spontaneous healing of the lesions and 2–5% develop MCL (Koff and Rosen, 1994). *Leishmania guyanensis* infection also is associated with multiple ulcers that can spread along the lymphatics and is known as pianbois. These lesions generally require treatment and often reoccur (Burza et al., 2018).

Visceral Leishmaniasis

Visceral leishmaniasis (VL) affects the spleen, liver, bone-marrow, and other visceral organs. There is no cutaneous pathology associated with initial presentation and clinical manifestations include persistent fever, hepatosplenomegaly, and weight loss. The disease can be either acute or gradual and is generally fatal within 2 years without treatment as a result of secondary bacterial infections or severe anemia. Acute malnutrition and high parasite burdens are present in young children with VL (Harhay et al., 2011). In the Indian subcontinent, hyperpigmentation of the skin is associated with VL, so is often referred as kala-azar, meaning black fever. Although endemic in 97 countries and territories, nearly 90% of the global burden of VL occurs in just 6 countries: Brazil, Ethiopia, India, Somalia, South Sudan, and Sudan (WHO, 2017).

Post-kala-azar dermal leishmaniasis (PKDL) is a late manifestation of VL caused by *Leishmania donovani* following treatment. PKDL presents as a hypopigmented macular or erythematous maculopapular rash on the face that can, in some instances, extend to the entire body. In PKDL, the parasites seem to persist in the skin after treatment. The syndrome can be mistaken for lepromatous leprosy but can be distinguished by the preservation of sensation (Burza et al., 2018).

Figure 5. Distribution of hunt clubs with confirmed cases of visceral leishmaniasis, United States and Canada. States in which hunt clubs or kennels had ≥ 1 dog infected with *Leishmania infantum* are shaded. *Leishmania*-positive foxhounds were also found in Nova Scotia and Ontario, Canada. Source: Duprey et al., 2006. Public domain.

Leishmania donovani.

The WHO estimates that over 70% of global VL caused by *Leishmania donovani* cases occurs in the Indian subcontinent and eastern Africa (WHO, 2016). Currently, East Africa has the highest burden of VL due to ongoing success with elimination efforts in Southeast Asia (Alves et al., 2018). VL caused by *Le. donovani* is considered anthroponotic because humans are the primary reservoir, although domestic dogs have been implicated as a possible minor reservoir host (Jambulingam et al., 2017).

The primary sand fly vector for VL in India and Bangladesh is *Phlebotomus arentipes*, and *P. orientalis* and *P. martini* for East Africa. In India the disease is associated with poor housing conditions where houses typically are made of mud walls and livestock and humans live under the same roof, creating an excellent ecological niche for the vector. VL in East Africa occurs primarily in arid and semi-arid lowland areas and is associated with migrant agricultural workers that typically sleep outdoors (Argaw et al., 2013).

Asymptomatic *Leishmania donovani* infections are common in endemic areas with seroprevalance in healthy individuals ranging between 7–63% in India (Srivastava et al., 2013) and 7–46% in Ethiopia (Ayehu et al., 2018; Abbasi et al., 2013). The underlying mechanisms that lead to clinical disease are not elucidated although malnutrition is thought to play a role and immunosuppression, particularly HIV coinfection in Ethiopia, is a major contributor. Commonly the incubation period is between 2 and 6 months and between 2–23% of asymptomatic individuals will present with VL symptoms within a year (Burza et al., 2018). VL caused by *Le. donovani* is almost always fatal unless treated and viable parasites can persist even after successful treatment, reactivating to cause disease if the individual becomes immunosuppressed (Diro et al., 2015).

Even without immunosuppression, PKDL can develop after apparently successful treatment. PKDL occurs in 25–50% of treated patients in Sudan within 6 months but is less common (5–10%) and occurs much longer after treatment (2–3 years) in India (Zijlstra et al., 2003). Interestingly, 5% of Indian PKDL patients report no previous VL episode (Zijlstra et al., 2016). In Asia, 90% of PKDL cases are of the macular type and African PKDL cases are primarily papular rash (Burza et al., 2018). Up to 85% of PKDL cases in East Africa are self-curing within 12 months and primarily pose aesthetic problems, although a small number will develop severely debilitating forms (Zijlstra et al., 2016). Important, however, is that PKDL lesions remain infectious for sand flies, serving as a reservoir of infection (Molina et al., 2017).

Leishmania infantum.

Leishmania infantum has a wide geographic distribution being endemic in the Americas, North Africa, the Mediterranean Basin, the Middle East, and Central Asia. Over 90% of VL cases due to *Le. infantum* occur in Brazil. VL was first discovered in the new world in 1937 and the parasite isolates were thought to be a new species and named *Le. chagasi* (Da Cunha et al., 1937). The following year, the discoverers realized that the parasites behaved like *Le. infantum* and concluded that the parasites that cause VL in the New World was identical to *Le. infantum* (Da Cunha, 1938), however the name *Le. chagasi* continued to be utilized in the literature. Modern molecular tools also are not able to distinguish *Le. infantum* from *Le. chagasi* (Mauricio et al., 1999), leading to the general agreement that the isolates from different geographical and host origins are, indeed, the same species. However, minor phenotypic and genotypic differences have led some authors to separate them into 2 species or, alternatively, 2 subspecies named *Le. infantum infantum* and *Le. i. chagasi* (Lainson and Rangel, 2005).

Infection with *Leishmania infantum* is primarily zoonotic, with the domestic dog as the reservoir host. Transmission occurs through the bite of sand flies of the genus *Lutyzomia* in the New World and *Phlebotomus* in the Old World. VL in Brazil used to be primarily restricted to rural areas in northeastern Brazil (De Melo and Fortaleza, 2013). Deforestation and associated changes in ecological habitats for the vector, urbanization, human migration, and the spread of HIV are changing the epidemiological profile to include urban epicenters of disease and a southward expansion (Arias et al., 1996). This changing epidemiological picture complicates prevention as measures directed at controlling the disease through vector control (insecticide spraying, use of repellents, or environmental management) or through management of canine leishmaniasis (dog culling or vaccination) are more difficult in urban settings (De Melo and Fortaleza, 2013). Moreover, control methods directed at controlling canine leishmaniasis have had varied outcomes (Romero and Boelaert, 2010).

As with *Leishmania donovani,* asymptomatic infections are common (9–24%) with individuals infected with *Le. infantum*. Conversely, there is little PKDL associated with *Le. infantum* infection except in cases of immunosuppression (Stark et al., 2006). *Leishmania infantum* can also cause LCL associated with single nodules and minimal inflammation that self-heal and induce immunity (Burza et al., 2018).

Visceral leishmaniasis (VL) caused by *Leishmania infantum* in the Old World continues to be primarily a rural disease, however a recent outbreak in Spain occurred in an urban area and was linked to a wild hare reservoir host (Arce et

al., 2013). Direct transmission without a sand fly vector also has been documented in Spain between intravenous drug users co-infected with HIV through needle sharing (Alvar et al., 1997).

During 2001–2016, 25 VL diagnoses were reported from United States soldiers deployed in the Middle East (Stahlman et al., 2017). Over the past decade, it has begun to be appreciated that asymptomatic VL is common in endemic regions, however the role of asymptomatic individuals in disease transmission and how many may progress to fulminant VL is less clear. Recently, higher risk United States military personnel were assessed 11 years after deployment in the Iraq War (2002–2011) to determine the rate of asymptomatic individuals; nearly 20% of these individuals were positive for *Leishmania infantum* infection (Modý et al., 2019). The risk of reactivation to VL for United States military veterans or to blood safety in the United States blood supply remains to be determined.

Canine Leishmaniasis

Zoonotic leishmaniasis can be found in all forms: Visceral, cutaneous, and mucocutaneous. However, canines act as the major reservoir of infection for *Leishmania infantum* which makes zoonotic visceral leishmaniasis (ZVL) the most pervasive of all forms of zoonotic leishmaniasis diseases (Gramiccia and Gradoni, 2005). Canine leishmaniasis (CanL) traditionally affects dogs in the same geographic regions as human visceral leishmaniasis such as the Middle East, South Asia, Central America, South America, North Africa, and East Africa (Tuon et al., 2008). Although dog ownership is not strictly necessary to place a person at higher risk of infection with leishmaniasis, regions with higher rates of CanL observe higher rates of human disease as well. In recent years, cases of CanL have been seen in non-endemic areas (Duprey et al., 2006).

Figure 6. Canine visceral leishmaniasis. Source: Dantas-Torres, 2008. License: CC BY 2.0.

In the United States, it was previously thought that CanL cases seen were strictly due to foreign travel. However, in the late 1990s, infections began to appear in the foxhound kennels (over 40% presented with disease) where foxhounds had no history of travel. A survey of the United States and Canada in the early 2000s found that 18 states and 2 provinces were enzootic for canine leishmaniasis (Duprey et al., 2006; see Figure 5). With no seropositive cases of *Leishmania* found in wild canines or in humans with close contact to the kenneled dogs, dog-to-dog transmission was considered to be the main route of infection. Risk factors associated with dog-to-dog transmission were thought to be the large number of animals housed together at a time, travel of foxhounds internationally and across state lines for breeding and club practices, and the inherent nature of the breed (Duprey et al., 2006).

Transmission.

As with human infections, *Leishmania* transmission to dogs principally occurs through the bite of phlebotomine sand flies through most of the world. Old World transmission occurs through the bite of *Phlebotomus* spp. sand flies, and *Lutzomyia* spp. sand flies in the New World (Killick-Kendrick, 1999). The sand fly vector appears to have preferential feeding on short-haired canines as higher rates of infection have been observed in short hair breeds (Dantas-Torres, 2008; França-Silva et al., 2003). Dogs receive bites in hairless areas such as the ear pinna, nose, and inguinal and perianal areas (Alvar et al., 2004). Although any dog is at risk for infection, dog breeds such as cocker spaniel, boxer, rottweiler, and German shepherd appear to be the most susceptible to infection (Killick-Kendrick, 1999). Thus far, very few breeds, like the Ibizian Hound, have shown resistance to developing clinical signs (Solano-Gallego et al., 2009).

Experimental studies have demonstrated that phlebotomine sand flies from non-endemic areas may become infected with *Leishmania* after feeding on an infected animal (Travi et al., 2002; van Griensven and Diro, 2019; Aronson and Joya, 2019). PCR analysis can deliver results with a quicker turnaround time (under 24 hours) and is able to detect low levels of parasitemia compared to the lengthier traditional methods

of diagnosis (Srivastava et al., 2011; Aronson et al., 2017; Akhoundi et al., 2017). However, PCR-based assays can still be disadvantageous for many endemic regions as the techniques are generally more expensive and complex than microscopy, require well trained staff, and require more financial resources to maintain equipment and purchase reagents (Burza et al., 2018; Vink et al., 2018).

Loop mediated isothermal amplification (LAMP) assays may be the answer to keeping all the advantages of molecular techniques while also reducing the associated disadvantages. In LAMP assays, there is no longer a need for expensive equipment as DNA amplification occurs at a constant temperature (~ 60 °C), reagents are lyophilized, and the product is easily visualized with simple methods (Mukhtar et al., 2018). Currently, the Loopamp *Leishmania* Detection Kit™ (Eiken Chemical Company, Japan) is available on the market and has shown promise for point-of-care testing with multiple sample types for several *Leishmania* species and assessment of cure for VL and PKDL (Vink et al., 2018; Mukhtar et al., 2018; Verma et al., 2017).

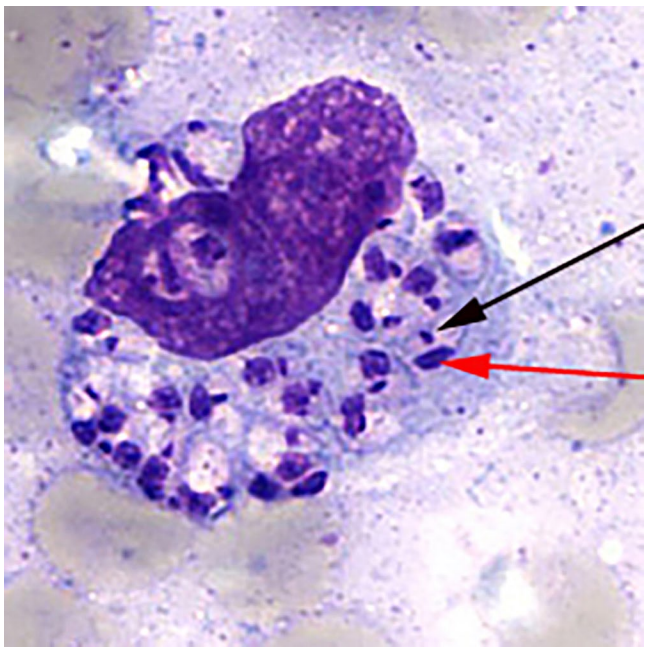

Figure 7. Light-microscopic examination of a stained bone marrow specimen from a patient with visceral leishmaniasis—showing a macrophage (a special type of white blood cell) containing multiple *Leishmania* **amastigotes** (the tissue stage of the parasite). Note that each amastigote has a **nucleus** (red arrow) and a rod-shaped **kinetoplast** (black arrow). Visualization of the kinetoplast is important for diagnostic purposes, to be confident the patient has leishmaniasis. Source: United States Centers for Disease Control and Prevention, DPDx. Public domain.

Treatment

Visceral Leishmaniasis

Many of the treatments for VL are toxic and/or expensive. The use of the different drugs, dosage, and treatment regimens differs depending on the *Leishmania* species initiating the infection, the geographical location, associated drug resistance in the region, and HIV co-infection. For current guidelines consult Aronson et al. (2017) and Burza et al. (2018).

The heavy metal antimony was first introduced as a therapy for leishmaniasis in the 1940s and for decades pentavalent antimonials were used as a monotherapy. Currently there are 2 antimony formulations in use: Sodium stibogluconate marketed as Pentostam® from Glaxo-Smith Kline and megalumine antimoniate marketed as Glocontime® from Rhone-Poulenc. These drugs have poor oral bioavailability so are delivered either by intramuscular injection or intravenously at a dose of 20 mg/kg per day for 16–28 days. The treatment is painful to administer and there are many adverse symptoms associated with the treatment, including pancreatitis, hepatotoxicity, cardiotoxicity, and induction of arrhythmias (Sundar and Chakravarty, 2010). Drug resistance is emerging to sodium stibogluconate on the Indian subcontinent so is no longer recommended as a therapy in this area (WHO, 2010; Sundar et al., 2000).

Miltefosine (hexadecylphosphocholine) was introduced as a chemotherapeutic to treat visceral leishmaniasis in 2002 as a result of a special public-private program initiated by the WHO with the pharmaceutical company Asta Medica to repurpose the anti-cancer compound (Sunyoto et al., 2018). Miltefosine is a broad spectrum anti-microbial, originally developed as an anti-cancer agent in the 1980s but adverse events in several phase I and II clinical studies resulted in the discontinuation of the drug's development as an oral anti-cancer drug (Planting et al., 1993; Verweij et al., 1993). As the only oral drug available to treat leishmaniasis, its introduction was seen as a breakthrough treatment. Unfortunately, this compound, marketed as Impavido® never reached its potential in controlling leishmaniasis due to gastrointestinal side effects, emergence of drug resistance, and cost (Dorlo et al., 2014; Ostyn et al., 2014; Rijal et al., 2013; Sundar et al., 2012). An economic analysis concluded that to be an effective public health tool, the drug should cost no more than US$ 50–60 per treatment (den Boer et al., 2011); currently the price for the public or non-for-profit sector in developing countries is US$ 117–160 and set at a market price of US$ 33,000–51,000 in the United States (Sunyoto et al., 2018).

Injectable paromomycin was introduced to combat leishmaniasis in 2006 and is used in combination treatment regimens with both pentavalent antimonials and miltefosine (Burza et al., 2018). The low cost of this drug (~ US$ 10–15)

is of great benefit to the public health sector. Amphotericin B deoxycholate, an anti-fungal drug, and its liposomal formulation are also used as monotherapy and in combination with other drugs.

Cutaneous Leishmaniasis

Most CL lesions will spontaneously heal within 2–18 months (David and Craft, 2009). For LCL caused by *Leishmania major* and *Le. mexicana* where 70% and 88%, respectively, of cases heal within 4 months, no treatment may be warranted. Treatment may be conducted to accelerate healing to reduce scarring, to reduce the risk of dissemination, or if the lesions are on the face or joints (Hodiamont et al., 2014; Weina et al., 2004). The Infectious Diseases Society of America and American Society of Tropical Medicine and Hygiene Clinical Practice Guidelines (Aronson et al., 2017) recommend local/topical therapy for non-healing simple lesions and systemic therapy for complex (MCL, RCL, DCL, DsCL, multiple lesions, lesions on face) and suggest that if resolution is apparent, management can occur without treatment if the patient concurs.

Treatment of CL has traditionally been administered by intralesional injection of the aforementioned drugs or topical application of antimicrobials such as paromomycin with or without methylbenzethonium chloride or gentamycin. Cryotherapy, a stimulus that decreases the lesion tissue temperature and results in cryonecrosis, has also been utilized (López-Carvajal et al., 2016). Recently, thermotherapy has been reintroduced as a therapy as amastigotes are heat sensitive and devices that deliver the radiofrequencies that deliver a temperature of 40–42 °C are relatively inexpensive (Burza et al., 2018). Systemic treatment is generally reserved for immunocompromised patients, individuals with multiple or refractory lesions, or patients at a risk for developing MCL (Burza et al., 2018; Aronson et al., 2017).

Table 2: Clinical Staging of Canine Leishmaniasis, Therapy and Prognosis

Clinical stage	Serology[a]	Clinical signs	Laboratory tests	Therapy	Prognosis
Stage I mild disease	Negative to low antibody levels	Mild clinical signs such as peripheral lymphadenopathy or papular dermatitis	Usually no clinicophathological abnormalities observed; normal renal profile	Allopurinol alone or with meglumine animoniate or miltefosine	Good
Stage II moderate disease	Low to high[b] antibody levels	Stage I signs plus diffuse or symmetrical cutaneous lesions such as exfoliative dermatitis/ onychogryphosis ulcerations (planum nasale, footpads, boy prominences, mucocutaneous junctions), anorexia, weight loss, fever, and epistaxis	Clinicopathological abnormalities such as mild non-regenerative anemia, hypergammaglobulinemia, hypoalbuminemia, serum hyperviscosity syndrome; normal renal profile [blood creatinine levels < 1.4 mg/dl] and non-proteinuric [urine protein to creatinine ratio (UPC) < 0.5] or UPC = 0.5–1	Allopurinol with meglumine animoniate or miltefosine	Good to guarded
Stage III severe disease	Medium to high antibody levels	Stage I & II signs plus may present vasculitis, arthritis, uveitis, or glomerulonephritis	Clinicopathological abnormalities from Stage II and chronic kidney disease (CKD) with UPC>1 or creatinine 1.4–2.8 mg/dl	Allopurinol with meglumine animoniate or miltefosine and follow guidelines for CKD	Guarded to poor
Stage IV extreme disease	Medium to high antibody levels	Stage I, II, & III signs plus pulmonary thromboembolism or nephrotic syndrome and end stage renal disease	Clinicopathological abnormalities from Stage II and creatinine 2–5 mg/dl or > 5 mg/dl. Nephrotic syndrome (UPC > 5)	Allopurinol alone and follow guidelines for CKD	Poor

[a] Dogs with negative to low antibody levels are confirmed as infected with additional diagnostic techniques. [b]High antibody levels are a conclusive diagnosis and are detected through immunofluorescence antibody test (IFAT) or enzyme-linked immunosorbent assay (ELISA). Table modified from Solano-Gallego et al., 2017.

Animal Models and Immunology

Susceptibility and resistance to *Leishmania* infection are regulated by genetic determinants and animal models have been instrumental in deciphering these mechanisms (Blackwell et al., 2009). In addition, many immunological aspects of the disease have been elucidated through the use of animal models, including mice, hamsters, dogs, and non-human primates (Loría-Cervera and Andrade-Narváez, 2014).

Mouse Model

Due to the existence of multiple strains of inbred mice, the simplicity of maintaining large numbers, and the vast number of reagents available for murine systems, experimental leishmaniasis in mice has been the primary animal model utilized for leishmaniasis research. For VL in mice, infection is primarily introduced via intravenous injection of large numbers of parasites. Two primary genetic loci, *Slc11a1* (also known as *Lsh/Bcg/XXX*) and *H2* [Major Histocompatibility Complex (MHC)], are associated with resistance to *Leishmania donovani* in mice. *Slc*11, the gene that encodes the transporter Nramp is responsible for resistance to *Le. donovani*, *Mycobacteria*, and *Salmonella* (Bellamy, 1999). In mouse strains that express the wild-type Nramp (for example, CBA mice), *Le*. *donovani* proliferation in the liver is inhibited. In strains that express a mutant Nramp (for example, BALB/c and C57Bl/6), parasite growth is unrestrained (Bellamy, 1999). The resistance mechanism only manifests in the initial stages of infection and MHC alleles that ultimately dictate adaptive immune responses can override Nramp susceptibility by inducing cure associated with reduced parasitic load in the liver. The non-cure mice progress to a chronic phase without clearing of the parasites.

The immune response to experimental VL and ultimate outcome of infection depends on the organ (liver or spleen) that is being assessed, the inoculation route and dose, and the parasite genotype (Loría-Cervera and Andrade-Narváez, 2014). Importantly, mice do not present the pathological features of human disease so are best used to determine infection susceptibility or resistance, rather than for assessing disease.

Rodents are a natural host for many cutaneous leishmaniasis causing species and so provide a good model for elucidating both immunological and genetic mechanisms of infection and pathology. Experimental infections with *Leishmania major* in particular have been instrumental in dissecting the immunological mechanisms of resistance (primarily C57Bl/6 strain) and susceptibility (primarily BALB/c strain) to infection and disease.

Resolution of cutaneous lesions in C57Bl/6 mice has been associated with a T-helper 1 (Th1) adaptive immune response that involves the production of interferon-gamma (IFN-γ) by CD4+ T-helper cells and stimulation of nitric oxide that is involved in destruction of amastigotes (Sacks and Noben-Trauth, 2002). Susceptibility in Balb/c mice correlates with a T-helper 2 (Th2) response characterized by the production of interleukin-4, interleukin-10, and transforming growth factor-beta (TGF-β) (Sacks and Noben-Trauth, 2002). The susceptible mice develop non-healing lesions and progressive disease, but not the clinical manifestations associated with VL. Genetic mapping assessing healing and non-healing phenotypes of progeny from crosses between resistant and susceptible mice revealed multiple genetic loci that influence both immune responses and wound healing (Sakthianandeswaren et al., 2009).

There are profound differences in the mechanisms that mediate susceptibility and resistance to infection and pathology associated with different *Leishmania* species. For example, C57Bl/6 and C3H mice that heal *Le. major* infections develop chronic disease when infected with either *Le*. *mexicana* or *Le*. *amazonensis* (Loría-Cervera and Andrade-Narváez, 2014). Chronic lesions due to *Le*. *amazonensis* are not dependent on a Th2 phenotype (Afonso and Scott, 1993) and parasite burden and pathology is exacerbated by Th1 cells (Soong et al., 1997). On the other hand, the non-healing phenotype of *Le*. *mexicana* infections in C57Bl/6 mice is associated with a Th2 response (Satoskar et al., 1995). There is no mouse model that recapitulates MCL caused by any *Leishmania* species so this system has had limited utility in understanding the pathology associated with MCL.

Hamster Model

The Syrian golden hamster (*Mesocricetus auratus*) is considered the best experimental model to study visceralizing *Leishmania* species (*Le*. *donovani* and *Le*. *infantum*) because this species is highly susceptible and reproduces the clinical pathology associated with visceral disease in humans (Melby et al., 2001). However, the dearth of immunological reagents (for example, antibodies for cell markers and cytokines) has hindered full understanding of the mechanisms of immunity.

Most studies in hamsters involve injection of large numbers of parasites via intravenous, intracardial or intraperitoneal injection that does not mimic the natural route of infection. Progressive disease involves uncontrolled parasite replication in the liver, spleen, and bone marrow despite the induction of T-helper 1 cytokines. Failure to control VL is associated with an immune suppression response associated with the production of TGF-β that triggers lymphocyte apoptosis (Banerjee et al., 2011), a lack of nitric oxide, the cytotoxin known to be required for killing of parasites (Pérez et al., 2006) and an inability of macrophages to process and present antigens to T-cells (Rodrigues et al., 1992).

Dog Model

Wild canines and domestic dogs serve as the primary reservoirs of zoonotic *Leishmania infantum* infection so the use of dogs as a model has recently gained momentum both to understand human VL and to identify mechanisms to treat canine VL. The primary mechanisms of protective immunity to *Le. infantum* in dogs is the activation of macrophages by a Th1 immune response (Vouldoukis et al., 1996). Canine VL is a multisystemic disease with variable clinical manifestations. Studies indicate a mixed cytokine response in CanL (Loría-Cervera and Andrade-Narváez, 2014). However, studies on experimentally infected dogs have shown that asymptomatic or resistant dogs produce a cell-mediated immune response to parasite antigens and more T-helper 1 associated cytokines than symptomatic dogs (Pinelli et al., 1994). The continued use of the dog as a model to study VL has the possibility of better understanding this complicated disease (see Table 2).

Non-Human Primate Models

Non-human primates are valuable models of human disease because of their similarities to human physiology and immunity. However, they are expensive and difficult to obtain and maintain, thus limited studies employ this model.

The Asian rhesus macaque (*Macaca mulatta*) is quite susceptible to *Leishmania* infection and the progression of CL and immune responses mimic CL in humans (Loría-Cervera and Andrade-Narváez, 2014). New World owl monkeys (*Aotus trivirgatus*) develop VL when infected with *Le. donovani* and exhibit weight loss, anemia, and hepatosplenomegaly similar to humans (Broderson et al., 1986). In contrast, squirrel monkeys (*Saimiri sciureus*) develop VL when infected with *Le. donovani* but recover and are resistant to reinfection (Dennis et al., 1986). Both symptomatic and asymptomatic *Le. donovani* infections are detectable in vervet monkeys (*Chlorocebus pygerythrus*) (see Gicheru et al., 1995) and Indian langurs have been used in vaccination studies (Misra et al., 2001).

Although the development of a non-human primate model that mimics human VL would certainly help elucidate the mechanisms of pathogenesis and immunity in humans, due to financial and ethical reasons, these models are typically only used when another model is not sufficient to answer a particular research question or as a final test for vaccines and drugs developed using other animal models.

Sand Flies

There has been partial success utilizing animal models for vaccine development against leishmaniasis; however, there still has been no licensure of an efficacious vaccine for humans. This general failure has led scientists to posit that the animal models should more closely mimic a natural infection (Reed et al., 2016). Sand flies are not simply syringes that inoculate parasites; rather they are a sort of pharmacy, capable of dispensing a plethora of pharmacologically active compounds into the skin of their hosts. These inoculated molecules have profound effects on the host immune system, exhibiting anti-haemostatic, anti-inflammatory, and immunosuppressive activities that facilitate blood feeding, while enhancing the establishment of the pathogens they transmit (McDowell, 2015). Moreover, *Leishmania*-infected sand flies also deposit a parasite-derived molecule, promastigote secretory gel (PSG), that accelerates wound healing while simultaneously enhancing survival and growth of *Leishmania* parasites (Giraud et al., 2018). To mimic a natural route of infection, many studies now incorporate sand fly saliva in the challenge inoculum and it has been posited that utilization of sand flies to inoculate parasites in the laboratory setting would advance vaccine development (Reed et al., 2016).

Inoculation of *Leishmania* parasites in the presence of sand fly saliva leads to enhancement of disease in animal models (Belkaid et al., 1998; Bezerra and Teixeira, 2001; Norsworthy et al., 2004; Samuelson et al., 1991; Theodos et al., 1991; Titus and Ribeiro, 1988). On the other hand, sand fly saliva has powerful immunogenic molecules that elicit strong hypersensitivity reactions in individuals that are repeatedly bitten. Repeated exposure to sand fly bites causes a delayed-type hypersensitivity (DTH) response recognized by local human inhabitants as a painful skin disease called harara (Adler and Theodor, 1957). Elicitation of this response has been suggested to be an evolutionary advantage for sand flies, by increasing blood-flow at the bite site and, therefore, decreasing the amount of time it takes for a sand fly to take a full blood meal (Belkaid et al., 2000). Although advantageous for sand flies, the DTH elicited by repeated exposure to sand fly bites (Kamhawi et al., 2000) or salivary gland homogenate (Belkaid et al., 1998) inhibits *Leishmania* infection in animal models. Thus, the inflammatory processes induced by the bite influence immunity to *Leishmania* parasites that are co-delivered with salivary components, effectively limit the ability of the parasite to cause devastating disease.

The prevalence of *Leishmania* infected sand flies in the field is quite low. For example, assessment of the sand fly vectors in specific areas in Iraq revealed that the highest infection rate was 2.3% (Aronson et al., 2003). Individuals in these areas can receive from 100 to 1,000 bites in a single night; therefore, the amount of sand fly saliva that is injected far outweighs any *Leishmania* antigen that may be inoculated, suggesting that sand fly saliva could be utilized as a part of a multi-component anti-leishmanial vaccine (Reed et al., 2016).

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MYXOZOA

13

Myxozoa

Myxozoa (Subphylum)

Terrence L. Miller

Phylum Cnidaria

Subphylum Myxozoa

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Chapter 13

Myxozoa (Subphylum)

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Introduction

Images that often come to mind upon hearing or seeing the word Cnidaria are swarms of jellyfish following the sun around lakes in Palau or the colorful tropical coral reef ecosystems with their vast diversity of hard and soft corals, and anemones housing charismatic clownfish. Rarely do images of parasites come to mind. However, recent phylogenetic and protein expression analyses have revealed the diverse group of obligate endoparasites of the subphylum Myxozoa (Grassé, 1970) (once considered a phylum in its own right) are in fact morphologically simplified, although highly specialized, cnidarians (Atkinson et al., 2018; Collins, 2009; Shpirer et al., 2018; Zrzavy and Hypsa, 2003). The primary uniting morphological feature of this group is the presence of a nematocyst-like structure termed the polar capsule in the myxozoan descriptive literature, which contains the polar filament that fires off in the presence of a suitable host, similar to the firing of nematocysts in free-living cnidarians used to capture prey (Figure 1).

The Cnidaria contained only 10 parasitic species previously, all of which had free-living stages at some point in their life cycle. The impacts of this seemingly innocuous change in classification now result in the once relatively parasite-free Cnidaria, now consisting of approximately 20% obligate parasites of a wide range of vertebrate and invertebrate hosts (Atkinson et al., 2018). This newly recognized and unique adaptive diversification of endoparasitic Cnidaria reveals that they are incredibly diverse in their specializations and ecologies, and greatly affect aquatic animal health in wild and cultured animal production systems.

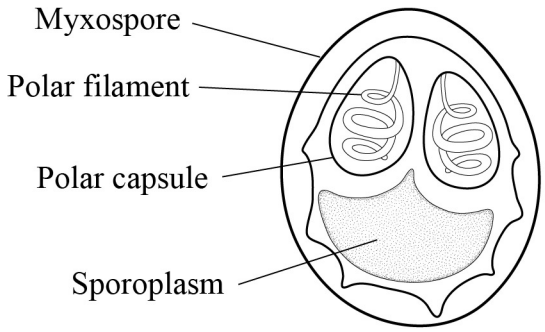

Figure 1. An uncharacterized species of *Myxobolus* (subphylum Myxozoa: class Myxosporea) found in *Acanthopagrus australis* from Moreton Bay, Australia illustrating the coiled nematocyst-like polar filament structure and general morphological characters. Source: T. Miller. License: CC BY-NC-SA 4.0.

Classification and Host Associations

The subphylum Myxozoa currently contains over 2,400 species in 2 disparately populated classes, the Malacosporea Canning et al., 2000, which currently consists of 2 genera (*Buddenbrockia* Schröder, 1910 and *Tetracapsuloides* Canning et al., 2002), and the Myxosporea Bütschli, 1881, which has over 60 genera (Atkinson et al., 2018). Generally, species within both classes require 2 hosts (an invertebrate as the definitive host and a vertebrate as the intermediate host) to complete their life cycle. The primary biological characteristics that distinguish the 2 groups are that malacosporeans use freshwater bryozoans and myxosporeans use marine or freshwater annelids as their invertebrate hosts (Patra et al., 2017). Species of both classes predominantly infect fish as their vertebrate host (> 95% of all reported species), however, a number of myxosporean taxa have now been reported from mammals, waterfowl, amphibians, and reptiles (Bartholomew et al., 2008; Hallett et al., 2015).

The fluid nature of taxonomic classification, especially above the level of the family, is demonstrated with respect to the Myxozoa due to the many hypotheses regarding the origin of these species and the nature of the evidence used to classify the group over the last couple of centuries. Their microscopic size and simple spore morphology led to the initial classification of myxozoans that were discovered in the early to mid-1800s as belonging to the Sporozoa, a group of unicellular spore-forming organisms in early literature (Okamura and Gruhl, 2015). Multicellularity of these organisms was identified in the late 19th century, but it wasn't until 1970 that the Myxozoa was formally considered a phylum in the Metazoa (Grassé, 1970; Štolc, 1899). Subsequent DNA sequence analyses of the nuclear small subunit ribosomal DNA

region (SSU rDNA) of myxozoans revealed their close phylogenetic relationships with the Cnidaria (Kent et al., 2001; Siddall et al., 1995).

Prior to 1994, classifications of the Myxozoa (that is, when it was then considered a phylum) contained 2 subclasses, the Myxosporea and Actinosporea, which were characterized based on distinctly different spore morphology and host associations (fish and annelids, respectively). It wasn't until experimental work investigating the transmission of *Myxobolus cerebralis* (the causative agent of whirling disease in salmonids) revealed that the markedly dissimilar spore morphologies observed in the annelid and fish hosts were actually just different developmental spore stages of the same organism (Markiw and Wolf, 1983; Wolf et al., 1986). Actinosporea was subsequently suppressed as a taxon (Kent et al., 2001; Kent and Margolis, 1994). Currently, DNA markers are primarily used (experimental infection trials less commonly) to determine conspecificity of actinospore and myxospore stages recovered from infected intermediate or definitive hosts (Hallett et al., 2002).

Life Cycle Stages and Infection Dynamics

Reproduction within the Myxozoa is as diverse as that observed within the free-living Cnidaria. Asexual and sexual reproduction in the Myxozoa are complex and detailed aspects of haploid and diploid cell formation within the group remains unknown. Evidence to date suggests meiosis generally occurs in the annelid or bryozoan host, which indicates these are the definitive hosts for these taxa (Okamura et al., 2015b). Transmission of myxosporean infections occurs through the production and release of actinospores from the annelid host and myxospores from the vertebrate host (Eszterbauer et al., 2015). Malacosporean transmission is similar in that infectious malacospores are released by the bryozoan host and the spores released from the fish host are characterized as fish malacospores (Hartikainen and Okamura, 2015; Patra et al., 2017).

Research into the causative agent of proliferative kidney disease (PKD), a significant disease impacting wild and cultured salmonids infected with *Tetracapsuloides bryosalmonae*, resulted in the original implication of bryozoans as hosts involved in the life cycle of this malacosporean pathogen (Hartikainen and Okamura, 2015). This and subsequent research into species of *Buddenbrockia*, which infects cypriniform and perciform fish, has contributed to the bulk of knowledge on the transmission and developmental stages of malacosporeans in their bryozoan and fish hosts (Hartikainen and Okamura, 2015; Patra et al., 2017). Malacospores in fish develop and mature in the kidney tubules and are released into the environment through urine excretion and infect the bryozoan host through ingestion of infectious spores or direct contact (Patra et al., 2017). Development within the bryozoan host results in the formation of multiple sac- or vermiform-like (termed myxoworm) stages containing many spores. These stages then burst inside of the bryozoan and spores are released into the water column via the vestibular pore (Hartikainen and Okamura, 2015; McGurk et al., 2006; Patra et al., 2017). Fish become infected when malacospores contact the gills or epidermis, the nematocyst-like polar filament everts to facilitate attachment, and the amoeboid sporoplasm invades the adjacent tissue (Hartikainen and Okamura, 2015).

Waterborne stages of individual myxosporean species released from the invertebrate and vertebrate hosts are often so morphologically dissimilar in appearance that, as discussed above, their original taxonomic classifications were unclear. The annelid hosts of myxosporeans vary depending on their occurrence in marine or freshwater environments. In freshwater ecosystems worldwide, oligochaetes are much more species rich than polychaetes (≈ 1,000 versus ≈ 170 species, respectively); whereas in marine ecosystems, oligochaetes are vastly outnumbered in species diversity by polychaetes (≈ 200 versus ≈12,000 species, respectively). Consequently, and possibly as a byproduct of coevolutionary radiation, the known annelid hosts of myxosporeans are primarily oligochaetes in freshwater and polychaetes in marine ecosystems (Alexander et al., 2015), although it should be noted that the life cycles are known for only very few species overall.

Infection of the annelid host is initiated via contact with infectious myxospores released from the vertebrate host (predominately fish). The site of infection in the fish host plays an important role in how myxospores are released into the environment. For example, species that form cyst-like pseudoplasmodia containing many infectious myxospores in various fish tissues (for example, in skeletal muscle, organs, or viscera), generally rely on the death of the host and post-mortem changes (decomposition or digestion) to facilitate spore release. For taxa present in marine ecosystems, direct predation or scavenging of fish harboring tissue-dwelling myxosporeans may be an important mode of viable spore release and spread in the environment through fecal material which settles in the benthos for exposure to their suitable annelid host. Species which develop mature spores in the biliary or urinary tracts of their fish hosts (for example, *Ceratomyxa* spp. or *Myxidium* spp.) are released directly into the environment through excretion of feces and urine.

Infection of the annelid host is considered to be primarily via ingestion of myxospores, although infection by direct contact with spores and amoeboid sporoplasms invading through the epidermis is likely (Alexander et al., 2015).

Once within the annelid host, the parasite then migrates to the preferred site of infection (for example, intestinal epithelium or body wall) and proceeds to undergo proliferative phases including schizogony, sporogony, and gamogony to produce numerous actinospores (Alexander et al., 2015; Eszterbauer et al., 2015; Hallett et al., 1999). Actinospores are then released into the water through the anus or epithelium of the annelid host by mechanisms which are still unclear (Alexander et al., 2015). Actinospore stages often have large caudal processes that presumably facilitate buoyancy and dispersal within the water column in order to come into contact with their suitable fish host. Infection of the fish host is through, 1) the anchoring of the actinospore to the gills or epithelium via the discharged polar filaments, and 2) the spore valves releasing the infectious sporoplasm, which 3) invades the adjacent epidermis and 4) subsequently migrates to the preferred site of infection (Kallert et al., 2015).

Diagnostics and Characterization

Diagnostic identification and taxonomic characterization of myxozoans via morphology only are complicated by the relatively few distinct morphological characters that can be used to discriminate between taxa. Most formal species descriptions of malacosporeans and myxosporeans are of the stages present in their vertebrate hosts, with taxa causing disease receiving particular attention. Descriptions of taxa generally incorporate characters such as **spore** size, shape, **pseudoplasmodia** dimensions and numbers of **spore valves**, **polar capsules** (including location and orientation), and coils of the **polar filaments** contained within being particularly important (Lom and Dyková, 2006; Patra et al., 2017). Other biological or ecological characteristics that are often important information accompanying species descriptions and aiding discrimination between taxa are the host species infected, tissue tropism, or site of infection. One of the most useful reviews and pictorial overviews for identification of myxozoan genera for someone new to the field is that of Lom and Dyková (2006; see Figure 2).

Currently, the inclusion of DNA sequence data as accompanying characters used to discriminate taxa are becoming critical for revealing taxonomic, ecological, and evolutionary relationships that were previously unresolved. The most common genetic markers used for comparative phylogenetic analyses and species-level distinction are the nuclear large and small subunit ribosomal DNA regions (LSU rDNA and SSU rDNA), which account for the majority of genetic data available on the publicly accessible databases GenBank and EMBL (Atkinson et al., 2015; Fiala, 2006). These ribosomal sequences contain a combination of highly conserved and variable regions, which correspond to the stem and loop motifs of the folded ribosome involved in the protein production machinery of eukaryotic cells. This combination allows for the robust alignment of conserved regions for phylogenetic or primer design purposes, and enough variability to reliably distinguish different sequence variants (Atkinson et al., 2015).

The consensus to date is that new species descriptions or revisions of taxonomic affinities within the Myxozoa should attempt to incorporate a combination of morphological characters (that is, examined via traditional microscopic and ultrastructural techniques), tissue tropism, host associations (including life cycle and host specificity data if possible), and DNA sequence data (Atkinson et al., 2015). The usefulness of these robust taxonomic treatments has been demonstrated through recent advances in knowledge of the biodiversity and ecology of myxozoans in aquatic ecosystems. Extraordinary species richness of myxozoans in aquatic environments is being revealed through recent biodiversity surveys, with some estimates suggesting the species diversity of myxozoans exceeds that of the number of fishes present in these ecosystems (Gunter and Adlard, 2008; 2009; Heiniger et al., 2011).

Aquatic Animal Health Implications

Much understanding of the biology, life cycles, and transmission dynamics of myxozoans has been prompted by investigations into the severe pathology and disease elicited by some myxozoan taxa, which cause significant negative economic and population-level impacts in aquatic wildlife and aquaculture. A few of the major diseases or production issues due to myxozoan infections are mentioned here to illustrate these impacts.

Proliferative Kidney Disease Caused by the Malacosporean *Tetracapsuloides bryosalmonae*

Proliferative kidney disease (PKD) is a condition which results in significantly high mortality rates in salmonid fish in Europe and North America caused by infections with the malacosporean *Tetracapsuloides bryosalmonae* (Canning et al., 1999). This disease is characterized by marked immunosuppression of the host, the proliferation of the parasite in kidney interstitia resulting in chronic hyperplasia and granulomatous reactions that cause distinct splenomegaly, renomegaly, and pathology in affected renal tissues (Sitjà-Bobadilla et al., 2015). Freshwater bryozoans of the class Phylactolaemata have been identified as hosts of this species. Infections in both the bryozoan and fish hosts appear to be temperature dependent, with higher prevalence and intensities observed in warmer months (Jones et al., 2015). The combination of habitat loss and degradation, warming climate, and impacts of PKD have been implicated in the decline of vulnerable trout populations throughout their native ranges.

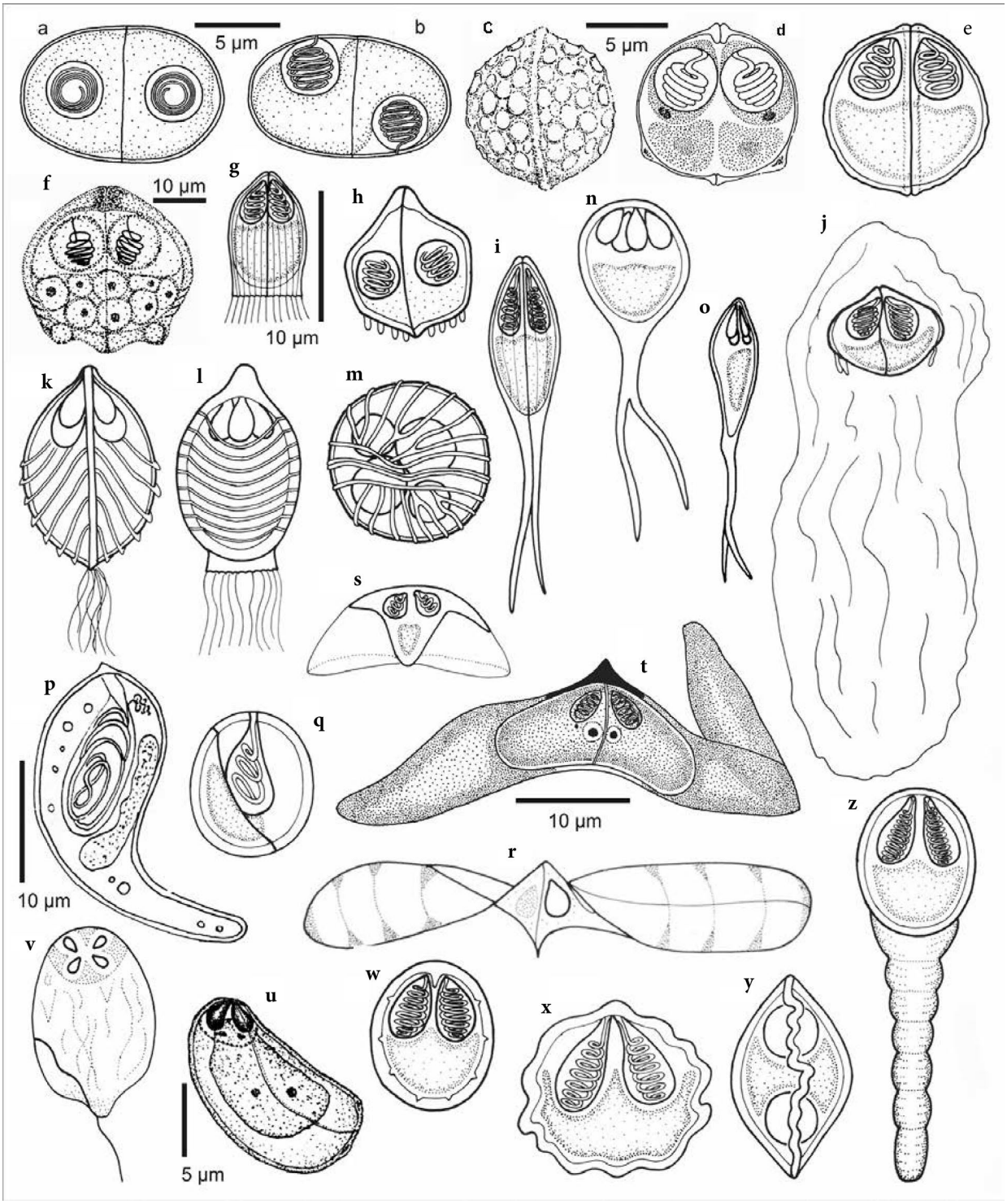

Figure 2. Line drawings of myxosporean spores. *Ellipsomyxa gobii* in (a) apical and (b) sutural view (adapted from Køie, 2003). *Sphaerospora elegans*, (c) pitted spore surface, (d) sutural view (Feist et al., 1991). e) *Sphaerospora renicola* in sutural view. f) *Polysporoplasma sparis* in sutural view (adapted from Sitjà-Bobadilla and Alvarez-Pellitero, 1995). g) *Hoferellus cyprini* in sutural view. h) *Wardia ovinocua*. i) *Myxobilatus gasterostei* in sutural view. j) *Palliatus mirabilis* in sutural view. *Chloromyxum leydigi* in (k) sutural and (l) frontal view. m) *Chloromyxum cristatum* in apical view. n) *Caudomyxum nanum*. o) *Agarella gracilis*. p) *Auerbachia anomala* (adapted from Meglitsch, 1968). q) *Globospora sphaerica*. r) *Alatospora samaroidea*. s) *Pseudoalatospora scombri*. t) *Renispora simae* (adapted from Kalavati, 1996). u) *Parvicapsula asymmetrica* (adapted from Shulman and Shulman-Albova 1953). v) *Neoparvicapsula ovalis*. w) *Myxobolus muelleri*. *Spirosuturia carassii* in (x) frontal and (y) apical view. z) *Unicauda clavicauda*. Sources: Lom and Dyková (2006) and adapted from sources noted in-line. License for all: CC BY.

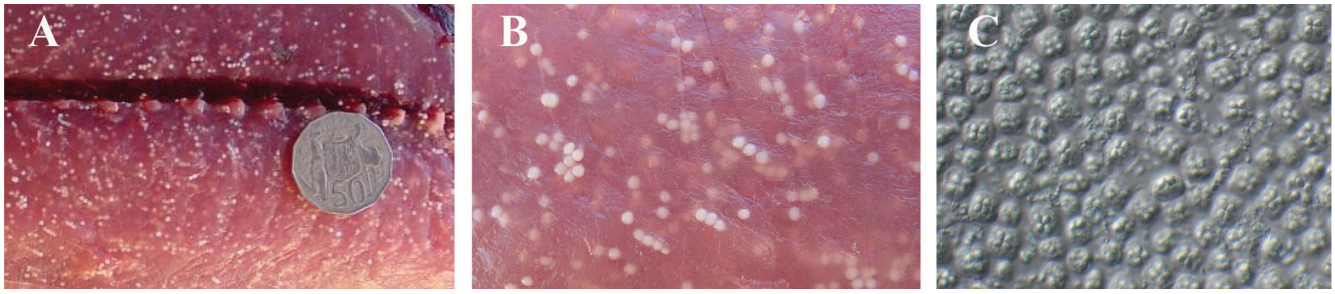

Figure 3. *Kudoa* sp. pseudoplasmodia in the flesh of a tuna collected off Ningaloo Reef, Western Australia. A) Macroscopic view with an Australian 50-cent piece for size reference B) Close up of macroscopic pseudoplasmodia in flesh, and C) Photomicrograph of spores released from pseudoplasmodia showing the 4 valves and 4 polar capsules characteristic of this this group. Source: T. Miller. License: CC BY-NC-SA 4.0.

Whirling Disease

Infections with the myxosporean *Myxobolus cerebralis* can cause the condition called whirling disease in salmoniform fish. As its name suggests, clinical signs of disease are characterized by the distinct erratic whirling patterns swum by affected individuals and a distinct darkening of the caudal region (Jones et al., 2015). *Myxobolus cerebralis* is one of the few known chondrophilic, or cartilage preferring, species. Development of the pseudoplasmodia primarily occurs in the cartilage of the head and vertebrae, and compression of the brain and medulla spinalis results in the abnormal swimming behavior observed in infected fish (Molnár and Eszterbauer, 2015). Discovery of the annelid host, *Tubifex tubifex*, as the obligate invertebrate host required to complete the life cycle, led to a revolution in understanding of myxosporean development and transmission (Jones et al., 2015).

Whirling disease has resulted in substantial decreases in susceptible salmonid populations throughout its known range, which is now considered distributed almost circumglobally wherever *Tubifex tubifex* is found. A notable exception is Australia, which is currently considered free of *Myxobolus cerebralis*. There it remains on the list of nationally notifiable diseases and strict quarantine measures are in force to reduce the possibility of incursion of this parasite, which could have devastating impacts to naïve salmonid populations on the relatively isolated island continent.

Myxosporean Infections and Seafood Marketability

In contrast to the myxozoan infections briefly mentioned in the above sections, some myxosporean species do not cause significant health issues or severe pathology in their host fish, but negatively impact the production and trade of seafood post-harvest. This is primarily due to the presence of unsightly macroscopic cyst-like pseudoplasmodia in flesh or myoliquefaction of infected musculature, external surfaces, or viscera. For example, a number of species of *Kudoa* are known to produce distinctly white, macroscopic pseudoplasmodia in muscle tissue of tuna species that stand out dramatically against the pink/crimson flesh of fresh fillets and render them effectively unmarketable for human consumption (Figure 3) (Moran et al., 1999). Another major marketability issue encountered in fish harvested from the wild or produced in aquaculture is post-mortem myoliquefaction, which results in mushy or butter-like consistency of fish muscle when cooked (Kristmundsson and Freeman, 2014; Langdon, 1991; Langdon et al., 1992; Moran et al., 1999). This is due to the presence of myxosporean pseudoplasmodia (often *Kudoa* or *Unicapsula* spp.) residing in myofibrils of infected fish releasing a suite of protease or proteolytic enzymes once the host has died, presumably an evolutionary adaptation to facilitate rapid release from the host into the environment (Alama-Bermejo et al., 2009; Lester, 1982; Stephens and Savage, 2010). Enzymatic breakdown of muscle tissue via this protease activity is accelerated in the presence of heat, not a particularly desirable combination when the product being marketed is destined for cooking prior to human consumption. High intensity infections can result in fish fillets subjected to heat via cooking displaying the consistency of jelly or peanut butter, which can elicit complaints from patrons visiting a restaurant or guests around the family barbecue.

Myxozoan infections and associated disease or marketability issues have had major negative impacts on wild fisheries and the aquaculture industries worldwide (Jones et al., 2015; Kent et al., 2001; Okamura et al., 2015a). Despite the significant progress made over the last few decades in the understanding of myxozoan biology, much remains unknown. From a biodiversity perspective, while around 2,400 species have been described, it appears that the surface has barely been scratched to discover the total myxozoan species richness of the world's freshwater and marine

ecosystems. In addition, less than 1% of all myxozoan species have had their complete life cycles elucidated and all susceptible hosts for particular species resolved. Further investigations into the diversity, host specificity, ecology, and transmission dynamics are clearly required to help mitigate the impacts of known and emerging diseases associated with myxozoan infections.

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MESOZOA

14

Mesozoa

Mesozoa (Phylum Dicyemida and Phylum Orthonectida)

Sarah R. Catalano

Phylum Dicyemida

Phylum Orthonectida

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Chapter 14

Mesozoa (Phylum Dicyemida and Phylum Orthonectida)

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Introduction

Unseen by the naked eye, the renal appendages (synonymous with kidneys, renal sacs, and renal organs) of benthic cephalopods, including squid, octopus, and cuttlefish (Figure 1), contain thousands of tiny worm-like organisms known as dicyemid mesozoans (Furuya and Tsuneki, 2003; Furuya et al., 2004; Finn et al., 2005). They can colonize 1 or both renal appendages at high numerical densities (Figure 2) and although simple in body structure, their life cycle is complex and not fully characterized. They are not known to infect any other marine organism, although the occurrence of a dispersal, free-swimming embryo form has led to questions as to whether an intermediate host or dormant stage exists.

Over 100 species have been formally described based on morphological characteristics, with documentation from the western and northeastern Pacific Ocean, northern Indian Ocean, Mediterranean Sea, northwestern and eastern Atlantic Ocean, Gulf of Mexico, Antarctic Ocean, and Southern Ocean. Only recently have molecular genetic analyses been applied to this group in an attempt to validate new species descriptions based on classical taxonomic methods and shed light on the unknown position in the Tree of Life for the enigmatic group of organisms.

Taxonomic Classification

Both the dicyemids and orthonectids (a group which parasitize a number of marine invertebrate phyla) have long been considered a class within the phylum Mesozoa (see Stunkard, 1972; Hochberg, 1983; McConnaughey, 1983a; 1983b).

Figure 1. The only known hosts of dicyemid mesozoans including A) squid (*Sepioteuthis australis*, southern calamari), B) octopus (*Octopus kaurna*, southern sand octopus), and C) cuttlefish (*Sepia apama*, giant Australian cuttlefish). Source: S. Catalano. License: CC BY-NC-SA 4.0.

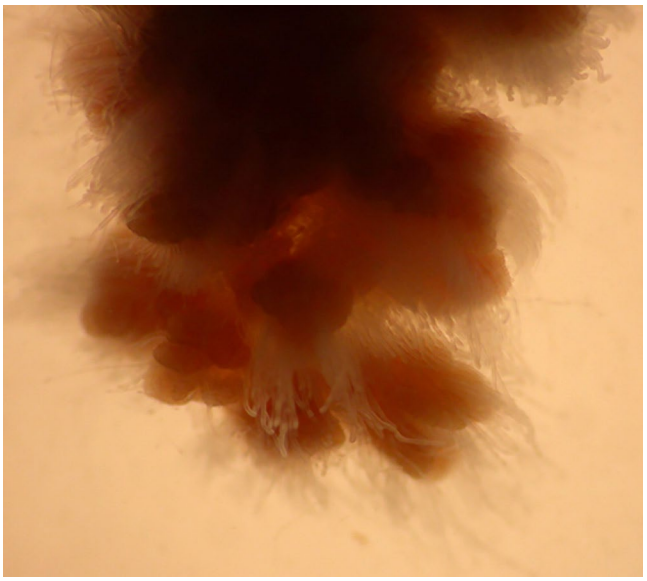


Figure 2. Hundreds of dicyemid mesozoans attached to the renal appendage (in red) of the giant Australian cuttlefish (*Sepia apama*). Each white strand represents 1 individual adult dicyemid. Source: S. Catalano. License: CC BY-NC-SA 4.0.

However, due to distinct differences between these 2 groups in terms of morphology and life cycle stages, it is now accepted to treat each group as separate phyla, phylum **Dicyemida** and phylum **Orthonectida**. The phylum **Dicyemida** contains 3 families, Dicyemidae Van Beneden 1882, Conocyemidae Stunkard 1937, and Kantharellidae Czaker 1994, although the validity of the Kantharellidae is questionable and uncertain due to the single species from this family being inadequately described (Furuya et al., 2007). Nine genera are recognized within the 3 families:

Family Dicyemidae
Dicyema von Kölliker, 1849
Dicyemennea Whitman, 1883
Dicyemodeca (Wheeler, 1897) Bogolopova, 1957
Pseudicyema Nouvel, 1933
Pleodicyema Nouvel, 1961
Dodecadicyema Kalavati & Narasimhamurti, 1980

Family Conocyemidae
Conocyema Van Beneden, 1882
Microcyema Van Beneden, 1882

Family Kantharellidae
Kantharella Czaker, 1994

The largest number of described species are in *Dicyema*, followed by *Dicyemennea*, with the other genera being monotypic or containing a small number of species (Catalano, 2012). Catalano (2012) provides a comprehensive list of the 112 species described up until 2012, however, an additional 12 species were described up until 2019 (Catalano, 2013a; 2013b; Catalano and Furuya, 2013; Castellanos-Martínez et al., 2016).

Typically for generic and species classification, the number and orientation of cells in each tier of the calotte, the presence or absence of abortive axial cells, the presence or absence of syncytial stages, the size of the adult stages, the number of cells comprising the body, the shape of the calotte, the anterior extension of the axial cell, the presence or absence of verruciform cells, and the structure of the infusoriform larvae are distinguishing morphological characters (Hochberg, 1982; 1983). However, recent molecular analyses have shed a level of doubt on some of these morphological characters, particularly calotte cell counts for genera classification, as the placement of a *Dicyema* species, with 4 metapolar cells in its calotte, grouped within the *Dicyemennea* clade, which is known to have 5 metapolar cells in their calottes (Catalano et al., 2015). Further molecular analyses, which include multiple species from all the known genera alongside additional molecular markers, will be needed to resolve and either validate or dismiss the current level of classification based on morphological traits. New species descriptions should now not only include measurements from all life cycle stages (nematogen, rhombogen, vermiform embryo, and infusoriform embryo) along with line drawings and light micrograph images of distinguishing characters (classical morphological measures, for example, as in Furuya, 2009), but also molecular analyses with the *COI* (*c* oxidase subunit I) gene sequenced as a minimum for inclusion in the preliminary phylogenetic tree for dicyemid mesozoans as presented by Catalano et al. (2015). Recently, Drábková et al. (2022) used several phylogenomic methods to generate a phylogeny that shows a common ancestor of the Dicyemids and the Orthonectida with ancestral Platyhelminthes as the basal group from which the Mesozoa arose.

Morphology

The body plan of a dicyemid mesozoan is very simple, comprising 8 to 40 cells with no body cavities, differentiated organs, tissues, or glands (Suzuki et al., 2010), although the dispersal embryo form, known as the **infusoriform embryo**, is morphologically distinct from the remaining 3 forms. The **vermiform adult**, **vermiform embryo**, and **rhombogen adult** (collectively known as the **vermiform stages**, Figure 3), all contain a central, long **axial cell**, which is where developing embryos are derived (Awata et al., 2006). This cylindrical axial cell is then protected by the presence of ciliated **peripheral cells** that surround the axial cell in a single layer, although at the anterior region, the peripheral cells are modified to form a **calotte**. The calotte serves as the dicyemid's anchor—it is inserted into the convoluted surface of the host renal appendage allowing the parasite to maintain a foothold while the remainder of its body hangs free in the surrounding environment obtaining nutrients, as seen in Figure 2 (Furuya et al., 2003a; 2007). Traditionally the number and arrangement of cells in the top 2 tiers of the calotte (known as the **metapolar** and **propolar cells**) has been used to assign new species into 1 of the 9 described genera (Figure 4). In particular, it has been reported that the dicyemids have 4 propolar cells, but different numbers of metapolar cells: *Dicyema* (4 metapolar cells, propolar cells opposite to metapolar cells), *Pseudicyema* (4 metapolar cells, propolar cells alternate with metapolar cells), *Dicyemennea* (5 metapolar cells), *Dicyemodeca* and *Pleodicyema* (6 metapolar cells), *Dodecadicyema* (6 metapolar cells plus 3 micropolar cells: Small cells which form the anterior tip of the calotte, only found in *Dodecadicyema* species) (Figure 4). The species of the family Conocyemidae are characterized by having no metapolar cells, but either parapolar cells or

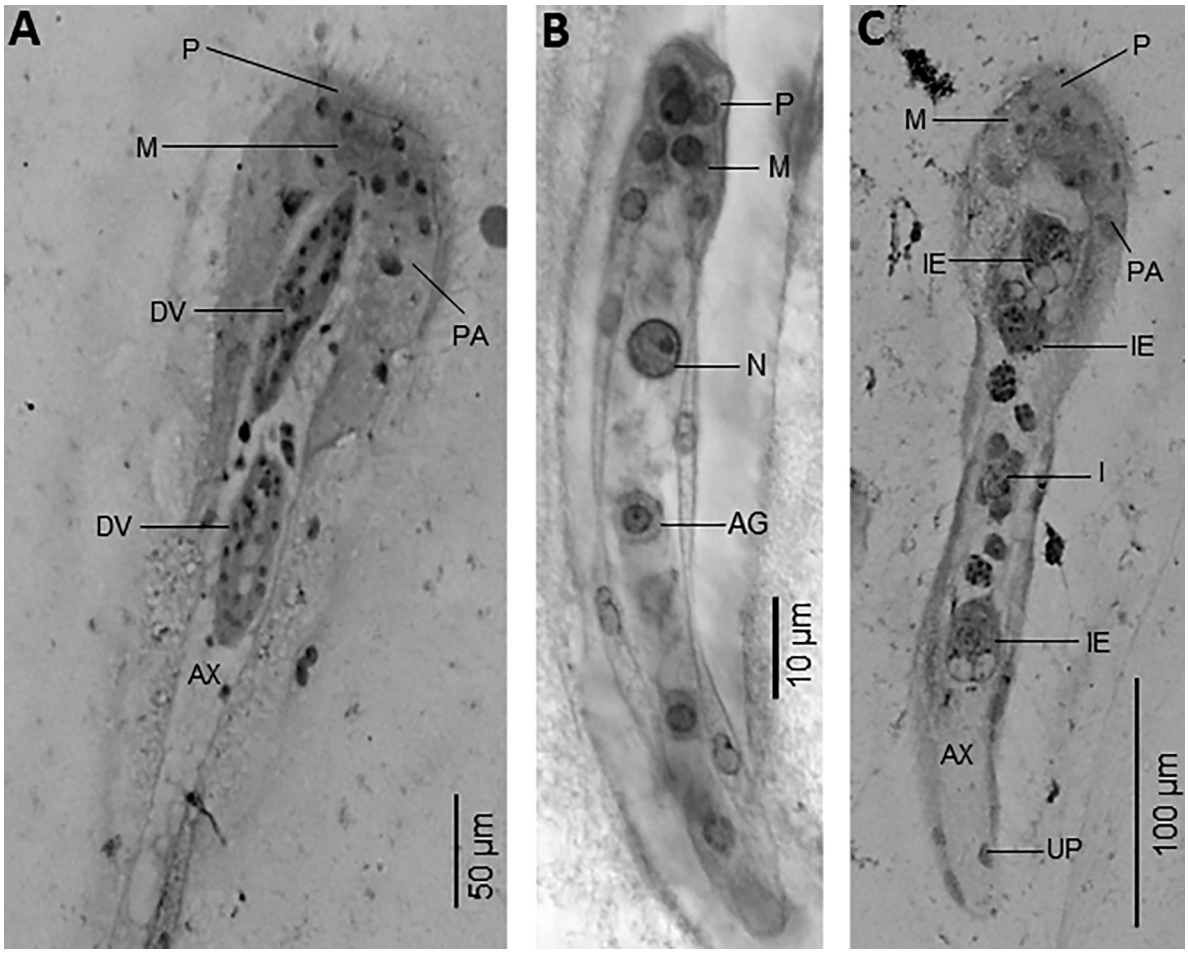


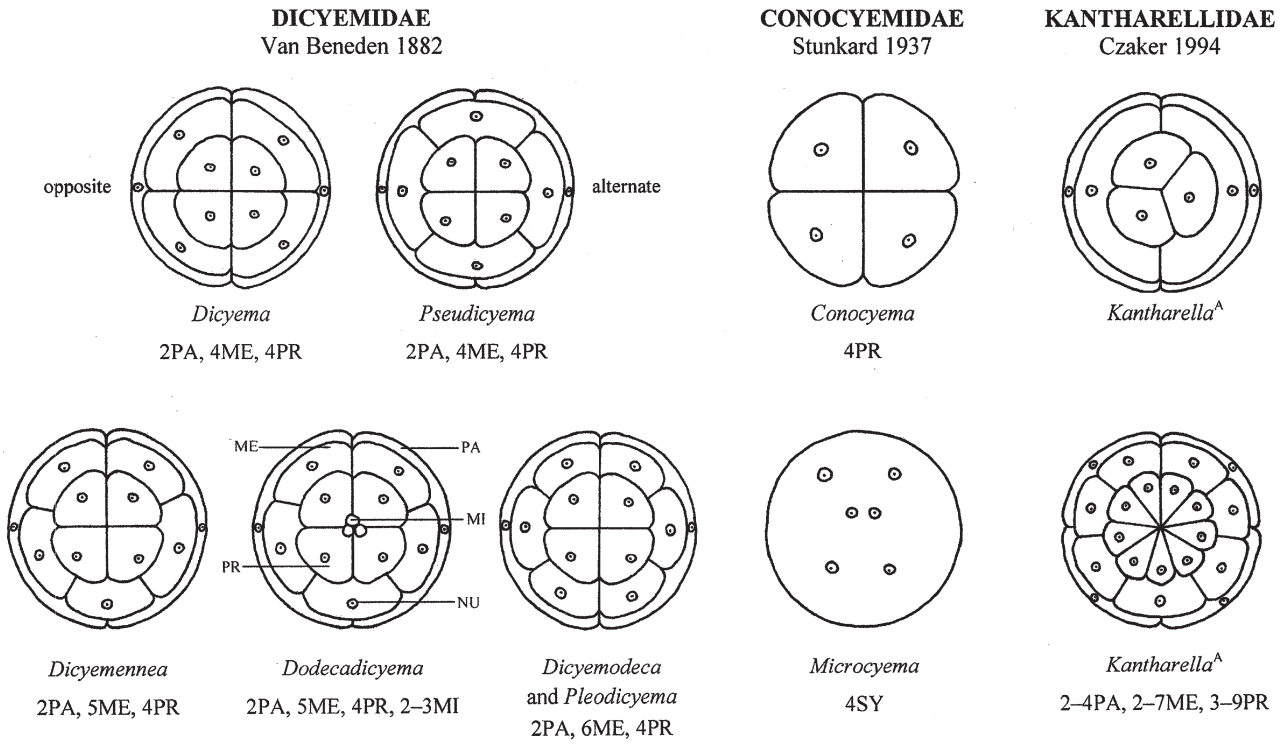
Figure 3. The vermiform stages: A) Adult nematogen (*Dicyema pyjamaceum*) with 2 developing vermiform embryos within the axial cell, B) close up of the vermiform embryo (*Dicyemennea floscephalum*) within the axial cell of a nematogen, C) adult rhombogen (*Dicyema pyjamaceum*) with 3 infusoriform embryos within the axial cell. Abbreviations: AG, agamete; AX, axial cell; DV, developing vermiform embryo; I, infusorigen; IE, infusoriform embryo; M, metapolar cell; N, nucleus; P, propolar cell; PA, parapolar cell; UP, uropolar cell. Adapted from Catalano and Furuya, 2013; Catalano, 2013a. Source: S. Catalano. License: CC BY-NC-SA 4.0.

a **syncytial cell**: *Conocyema* (4 parapolar cells) and *Microcyema* (syncytial cell: A single cell with 6 nuclei which is only found in *Microcyema* species) (Figure 4). The species of the Kantharellidae are different from the other families because there is no cell constancy, with 3 to 9 propolar cells, 2 to 7 metapolar cells, and 2 to 4 parapolar cells (Czaker, 1994) (Figure 4). Nonetheless, whether calotte cell counts represent an accurate way of distinguishing dicyemid genera remains in question, with molecular analyses shedding some doubt on this classical level of classification (see Catalano et al., 2015).

Unlike the vermiform stages, the infusoriform embryo is not long and cylindrical, but rather small and circular (Figure 5). The infusoriform embryo is characterized by 4 large internal **urn cells**, each containing a **germinal cell** which is thought to give rise to the next generation, as well as 2 large **apical cells** at the anterior region and beating **cilia** surrounding the body (Furuya and Tsuneki, 2003). One of the most interesting characters of the infusoriform embryo are the **refringent bodies**, which are contained within the large apical cells. These refringent bodies are composed of a chemical with a high specific gravity that accounts for more than one-third of the body weight of the infusoriform embryo, namely, **magnesium salt of inositol hexaphosphate** (Lapan and Morowitz, 1972; Lapan, 1975). This dense chemical provides the infusoriform embryo with negative buoyancy, which is suggested to allow the embryo to remain close to the sea floor to encounter and infect a new host.

Life Cycle

In contrast to their simple morphology, the life cycle of dicyemid mesozoans involves 2 stages of development



[A] = Alternative views of calotte arrangement for *Kantharella* with the minimum (top) and maximum (bottom) number of PA, ME and PR drawn.

Figure 4. Schematic drawing of anterior end views of calottes showing the arrangement and number of cells characteristic of different genera. Abbreviations: ME, metapolar cell; MI, micropolar cell; PA, parapolar cell; PR, propolar cell; SY, syncytial cell. Adapted from Catalano, 2012. License: CC BY-NC-SA 4.0.

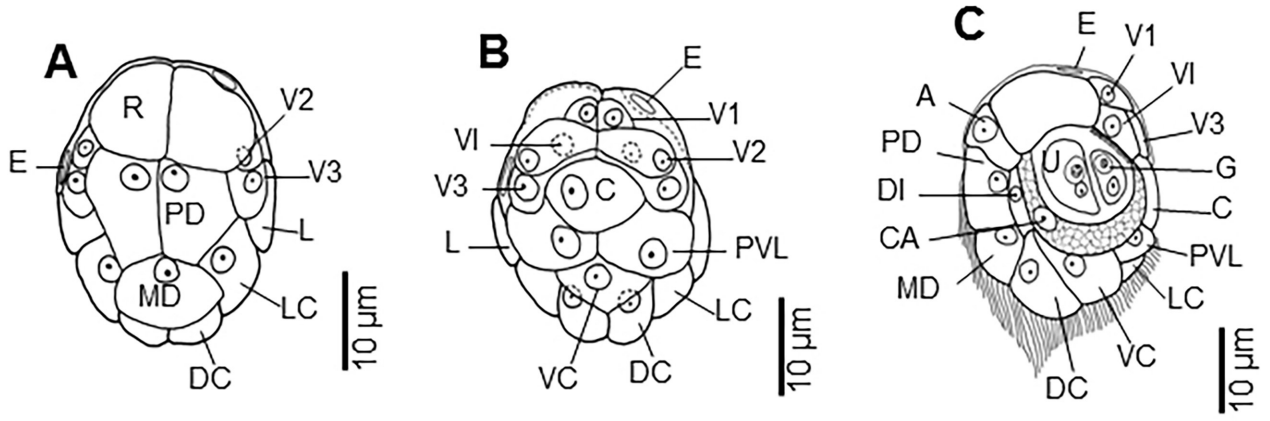


Figure 5. Line drawings of the infusoriform embryo (*Dicyemennea floscephalum*) showing A) dorsal view, cilia omitted, B) ventral view, cilia omitted, and C) sagittal section. Abbreviations: A, apical cell; C, couvercle cell; CA, capsule cell; DC, dorsal caudal cell; DI, dorsal internal cell; E, enveloping cell; G, germinal cell; L, lateral cell; LC, lateral caudal cell; MD, median dorsal cell; PD, paired dorsal cell; PVL, posteroventral lateral cells; R, refringent body; U, urn cell; V1, first ventral cell; V2, second ventral cell; V3, third ventral cell; VC, ventral caudal cell; VI, ventral internal cell. Source: Adapted from Catalano, 2013a. License: CC BY-NC-SA 4.0.

(vermiform and infusoriform) and 2 modes of reproduction (asexual and sexual) (Figure 6) (Furuya et al., 2003b; 2007). The vermiform stages, which are restricted to the renal appendages of the host, comprise the adult nematogen, vermiform embryo, and adult rhombogen. The infusoriform stage, which represents the dispersal stage that escapes from the host via the urine to find and infect a new host, comprises the infusoriform embryo.

While the vermiform stages are similar in terms of morphology, comprising 8–40 cells and a worm-like body shape, the infusoriform embryo is distinct, comprising 37–39 cells and being much smaller in size (typically 32–36 µm in length and 26–28 µm in width) with a rounded body shape (Furuya and Tsuneki, 2003). The vermiform stages are formed asexually from an agamete (axoblasts) whereas the infusoriform embryo develops from a fertilized egg produced around a hermaphroditic gonad called the infusorigen (Figure 7).

Within the axial cell of an adult nematogen, the vermiform embryos grow and develop asexually, with more than 1 embryo common within the single axial cell of the adult. Although the exact mechanism is unknown, the vermiform embryo is then released from the nematogen into the fluid around the renal appendages, finds a free surface for attachment and inserts its anterior calotte into the convoluted surface of the host's renal appendage while the rest of its body hangs free in the surrounding urine acquiring nutrients. As it develops further, it transitions into the adult nematogen and will produce its own vermiform embryo and the cycle continues. A high population density in the kidney, as seen in Figure 2—where an accumulation of a chemical factor in the environment is detected (Lapan and Morowitz, 1972; 1975)—is then thought to trigger a shift from asexual reproduction and increasing numbers within the renal appendage to a sexual mode of reproduction and escaping out of the crowded and highly infected host to find and infect a new, potentially naïve, host. This shift is seen in the form of production of the rhombogen adult in place of the nematogen adult, which contains in its single axial cell, 1 or more infusoriform embryos. These dispersal embryos then escape from the host out into the surrounding environment, ensuring that the species will survive beyond the eventual death of the host (Lapan and Morowitz, 1972).

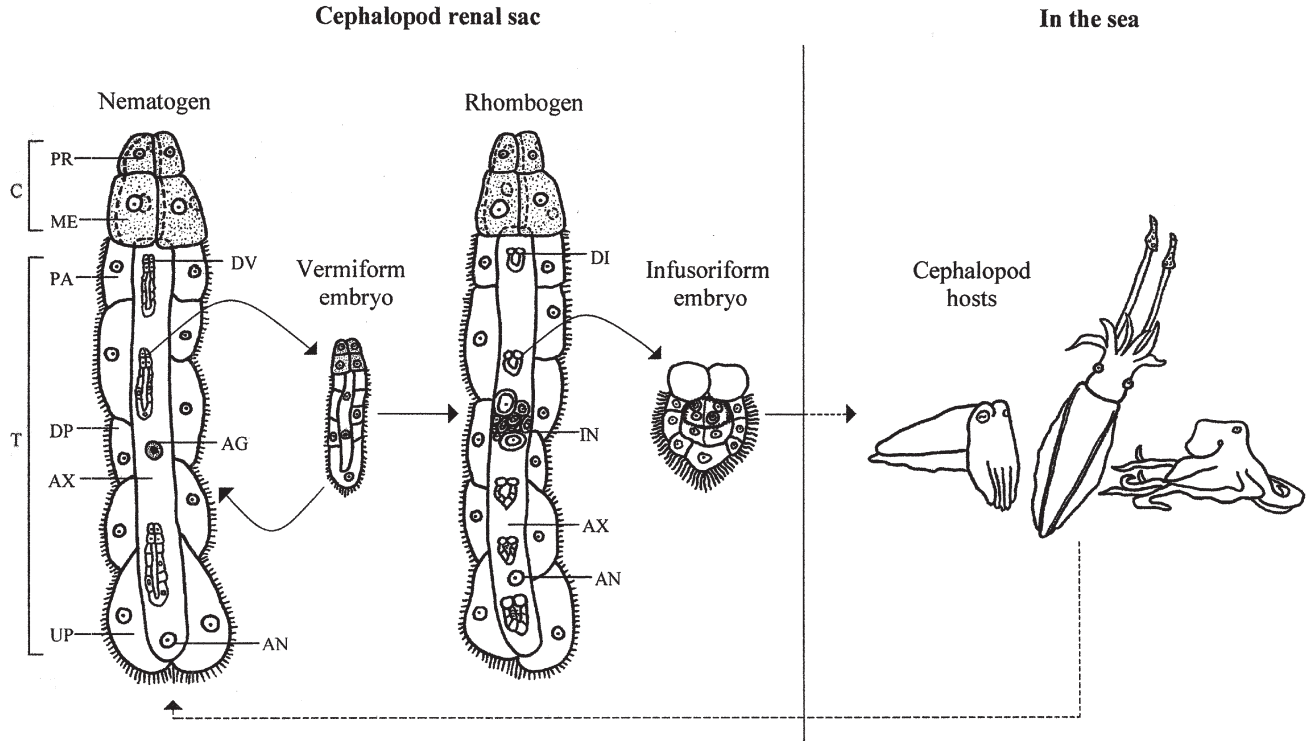


Figure 6. Diagrammatic representation of the morphology and life cycle of dicyemids. The dashed line indicates unknown processes of how the infusoriform embryo finds and infects a new cephalopod in the sea and how it then develops into adult forms. The nematogen, rhombogen, and vermiform embryo represent the asexual vermiform stages; the infusoriform embryo represents the sexual infusoriform stage. Abbreviations: AG, agamete; AN, axial cell nucleus; AX, axial cell; C, calotte; DI, developing infusoriform embryo; DP, diapolar cell; DV, developing vermiform embryo; IN, infusorigen; ME, metapolar cell; PA, parapolar cell; PR, propolar cell; T, trunk cell; UP, uropolar cell. Source: Adapted from Catalano, 2012. License: CC BY-NC-SA 4.0.

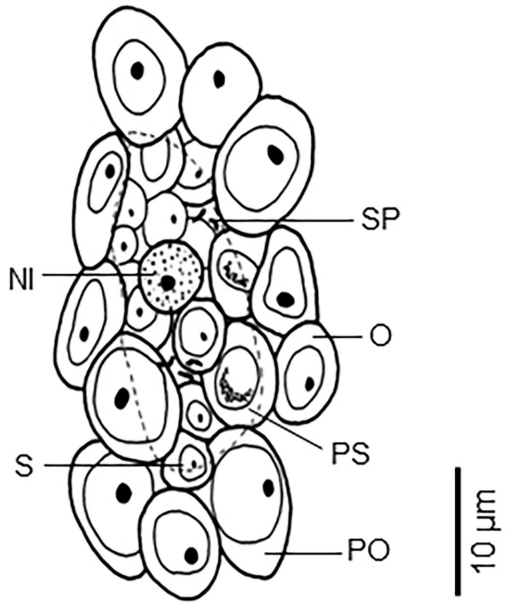


Figure 7. Line drawing of the infusorigen which is located within the axial cell of an adult rhombogen. Abbreviations: NI, nucleus of infusorigen; O, oogonia; PO, primary oocytes; PS, primary spermatocytes; S, spermatogonium; SP, sperm. Source: Adapted from Catalano, 2013a. License: CC BY-NC-SA 4.0.

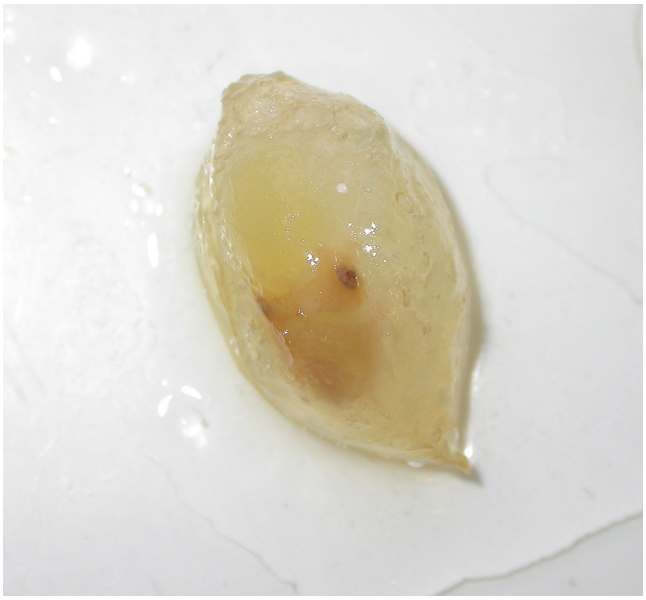


Figure 8. Host eggs have been hypothesized to be the potential stage of new infection for dicyemid mesozoans. Source: S. Catalano. License: CC BY-NC-SA 4.0.

The next stage of the life cycle remains unknown yet quite astonishing given most cephalopods are found to be infected by dicyemid parasites at high intensities. Particularly, it is uncertain how the tiny, infusoriform embryo, with limited swimming capabilities in relation to its host and a short survival time in seawater, then finds a new host, attaches to it or is taken up internally and starts the cycle off again by potentially morphing into the required vermiform stage (adult nematogen) so asexual reproduction can take place and the renal appendages of a new host will then become colonized.

Despite the monstrous challenge that the infusoriform embryo faces of finding and infecting the correct host species with a limited lifespan in a large, fluid, ever-changing environment, dicyemid parasites are still found in almost all benthic cephalopods examined (Catalano et al., 2014). As such, questions have been raised about an intermediate host; however, results from past experimental studies suggest the life cycle is direct (Lapan and Morowitz, 1975). Host eggs were hypothesized to be the potential stage of new infection, as they are abundant and sessile in the environment, allowing a huge number of new individuals to be infected with low energy costs (Figure 8) (Catalano et al., 2013). Additionally, as adult cephalopods have a short lifespan of 1 to 2 years, with mortality common after a single spawning event (Semmens et al., 2007), infection of the host egg stage provides dicyemids with the maximum amount of time for survival. Nonetheless, no dicyemid DNA was recovered from environmental water samples or cuttlefish eggs at the mass breeding aggregation of giant Australian cuttlefish (*Sepia apama* Gray) in South Australian waters (Catalano et al., 2013), leading to the notion that to resolve this unknown in the life cycle, experimental infection is needed. Interestingly, exclusive infection of the asexual stage of the dicyemid (adult nematogen) was found in the left renal appendage of a large giant Australian cuttlefish that had been held in captivity for 2–3 months, recently mated, and naturally died before samples were collected, indicating that dicyemids may persist and continue replicating even after host death (Catalano, 2013b). Furthermore, although the host had died, an immediate priority by the dicyemid was not to disperse, as density increased within the renal appendage with vermiform embryos continuing to produce instead of the dispersal of the infusoriform embryo (Catalano, 2013b). Perhaps a dead host just gets stuck with lingering parasites; or, perhaps, the life cycle of the dicyemid may be more intricate and mysterious than first thought.

Secondary Nematogens

Although not recognized as a regular part of the dicyemid life cycle, an additional form exists, namely the secondary nematogen. This rare form, which in the past has been denied to occur at all (Gersch, 1938), but has been observed by McConnaughey (1951) and Catalano (2013a), is characterized

by containing infusorigen and infusoriform embryos together with young vermiform embryos within the axial cell, in essence having features of both adult nematogens and rhombogens (Figure 9). This form is thought to result by accident in the transitional period of development from a nematogen to rhombogen, with persistence of some axoblasts in good condition through the rhombogen period that have been able to resume their activity and produce, once more, viable vermiform embryos (Catalano, 2013a; McConnaughey, 1951). Whether there is then the possibility for reversal back to a full nematogen form, brought about through competition between these 2 modes of reproduction, and the possibility of infusorigens becoming exhausted while axoblasts are still being produced, is unknown. It is also unknown whether the occurrence of secondary nematogens is species-specific (Catalano, 2013a).

Hosts and Patterns of Infection

The only recorded hosts of dicyemid mesozoans are cephalopods, which include squid, octopus, and cuttlefish (as seen in Figure 1). In general, dicyemid species are highly host-species specific, although typically, 2 or more species are recorded in each host species (Furuya, 1999; 2017). The common octopus, *Octopus vulgaris* Cuvier, has the largest number of dicyemid species recorded from it (11), followed closely by the stubby squid, *Rossia pacifica* Berry, with 9 species (see Catalano, 2012). Hochberg (1990) suggests that *O. vulgaris* and *R. pacifica* may actually represent a host species complex, each with their own distinct dicyemid fauna, and the reported parasites might make up a reciprocal species complex.

When more than 1 dicyemid species do co-occur within a single host individual, generally the calotte shape from each is different, allowing each dicyemid species to colonize a distinct niche or surface of the host renal appendage (Furuya et al., 2003a; Furuya, 2008; Furuya and Tsuneki, 2003). Species of dicyemids that possess similar calotte shapes are rarely found together in a single host individual (Furuya and Tsuneki, 2003). The microhabitat of the renal appendages provides all that the dicyemid requires to complete its life cycle, including a surface for attachment, constant fluid bath, a source of nutrients and a simple exit for release of the dispersal stage (Hochberg, 1982).

In general, the presence of dicyemids is more commonly observed in benthic rather than pelagic cephalopods, which has been related back to the negative buoyancy and sinking ability of the infusoriform embryo. However, other factors, such as host size, age, behavior, and geographic locality likely play a role in the presence of infection, as exceptions

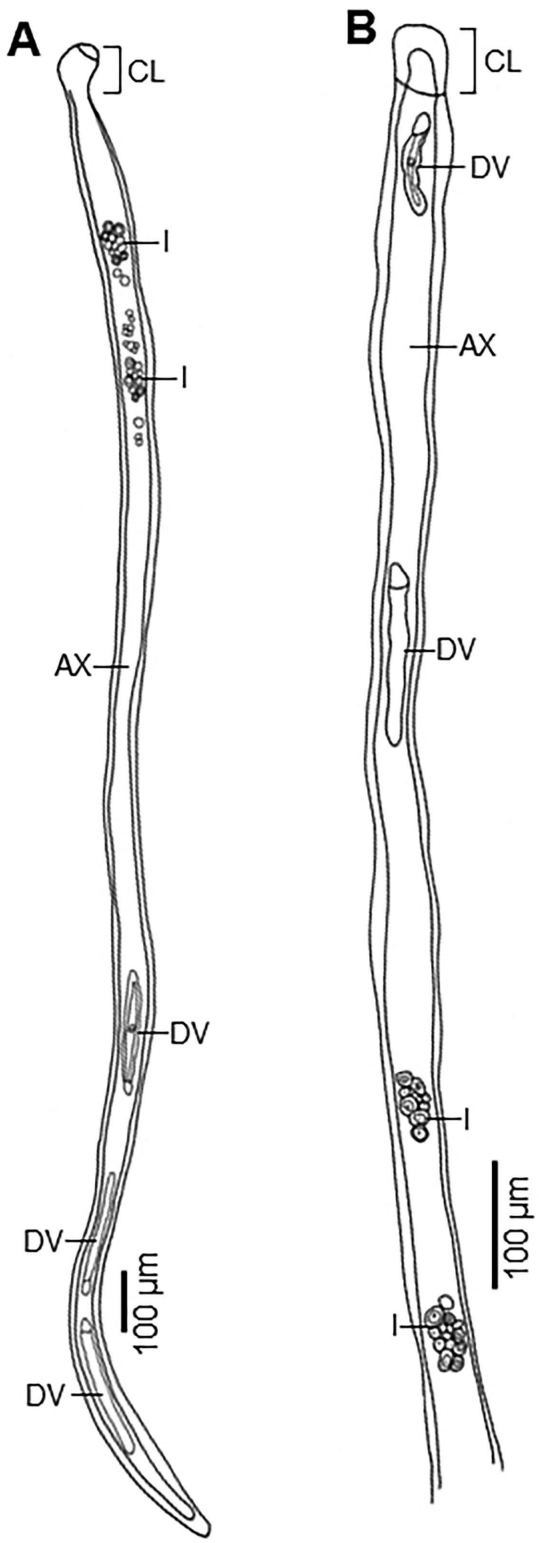


Figure 9. Line drawing of secondary nematogens (*Dicyema furuyi*) from *Sepia papuensis*. Abbreviations: AX, axial cell; CL, calotte; DV, developing vermiform embryo; I, infusorigen. Source: Adapted from Catalano, 2013a. License: CC BY-NC-SA 4.0.

to this notion are observed. For example, the southern dumpling squid, *Euprymna tasmanica* Pfeffer, frequently associates with the sea bottom, burying itself in the sand during the day to hide from predators (Norman and Reid, 2000). Such a strategy would allow for ample opportunity to be infected by the infusoriform embryo; however, in the study by Catalano et al. (2014), adding to the weight of evidence but while not conclusive, no dicyemids were recorded from this host species for 6 individuals collected and analyzed. In contrast, Finn et al. (2005) recorded the presence of dicyemids in 14 out of 18 *E. tasmanica* individuals from the same region, however, the 6 individuals collected in the study by Catalano et al. (2014) were small with a mantle length half of what is typically reached for this species. Other authors have also recorded absence of dicyemids from small host individuals (for example, Furuya et al., 1992b; Furuya and Tsuneki, 2005; Castellanos-Martínez et al., 2011). Furuya and colleagues (2004) correlated this to the complexity of the renal appendage, stating large host individuals have a more developed and complicated external surface compared to smaller host individuals, therefore they are able to provide more attachment sites and surface area for vermiform stages.

Interestingly within a single host individual, different stages (either exclusively the asexual stage or exclusively the sexual stage) have been recorded in each renal appendage, such as the adult nematogen (asexual stage) in the left renal appendage and the adult rhombogen (sexual stage) in the right renal appendage (Figure 10) (Finn et al., 2005; Catalano et al., 2014). This suggests that dicyemids infect the renal appendages independently of one another, at different times, and do not or cannot move from 1 renal appendage to the other. This also elucidates that the cue which mediates the transition from the asexual to the sexual stage is parasite driven rather than host-mediated, or else both renal appendages would be equally affected and contain the same stage at any one time (Lapan and Morowitz, 1975; Finn et al., 2005; Catalano et al., 2014).

True Parasite or Commensal Organism?

The true nature of the dicyemid mesozoan as a parasitic, commensal, or mutualistic organism remains unresolved with arguments for and against each option presented in the literature. Some authors support the parasitic way of life, stating that the delicate brush borders of the host renal appendage surface is damaged by the dicyemid attaching and maintaining its foothold, while others support the dicyemid as a commensal organism, stating they have little effect, either positive or negative, on the host (Finn et al., 2005; Furuya and Tsuneki, 2005). The third notion is that these organisms are

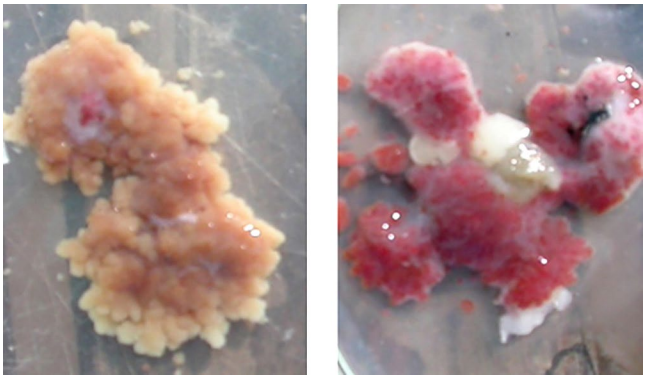


Figure 10. The left (brown) and right (red) paired renal appendages of cuttlefish, which can harbor different stages of dicyemid mesozoans in each renal appendage. Source: S. Catalano. License: CC BY-NC-SA 4.0.

mutualistic, with the beating cilia on their bodies facilitating host excretion of ammonia and urine, while also allowing the dicyemid to receive nutrients, taken up through the peripheral cells by endocytosis (Lapan, 1975; Hochberg, 1990; Furuya et al., 2004).

Molecular Analyses: Mitochondrial Mini-circle Molecules

Few studies have focused on the molecular genetics of the dicyemid mesozoans. For those that have reported molecular analyses, the mitochondrial (mt) cytochrome *c* oxidase complex unit genes (*COI*, *COII*, and *COIII*) have typically been sequenced, although some studies have reported sequences for nuclear genes from dicyemid species (Ohama et al., 1984; Katayama et al., 1995; Pawlowski et al., 1996). Quite unusually, the mt genome architecture of the dicyemids departs from the typical ~ 16 kb circular metazoan genome. In addition to a putative circular genome (Boore, 1999), the mt *COI*, *COII*, and *COIII* genes have been found to be located on separate mini-circle molecules, each with their own non-coding region (NCR) (Watanabe et al., 1999; Awata et al., 2005, Catalano et al., 2015). While the gene coding region defines genome metabolic functionality by specifying proteins, the NCR can define the architecture and regulation of the genome, often harboring the replication origin of the mini-circle and the promoters for transcription (Le et al., 2002; Burger et al., 2012). Although no specific origin of replication was found in the *COI* mini-circle molecules of 10 dicyemid species sequenced in the study by Catalano et al. (2015), palindrome sequences with the potential to form stem loop structures were identified in 5 species, suggesting that these palindrome regions may be involved in initiating mini-circle replication. Nonetheless it is quite

bizarre to have single mt genes on single mini-circle molecules, as the typical mt genome, where all the genes are linked together on a chromosome will ensure the complete genetic information is transmitted when the mitochondrion replicates. This is otherwise challenging with a fragmented mt genome structure as observed in the dicyemid mesozoans. Further molecular studies are needed for the dicyemid mesozoans, particularly to confirm the validity of classifications based on traditional morphological traits, with sequences of DNA containing thousands of characters with orders of magnitude more than morphological analyses (Poore and O'Hara, 2007).

Position in the Tree of Life

The position of the dicyemids in the Tree of Life has long fascinated researchers, with the Belgian biologist Édouard Van Beneden providing the first attempt to classify the dicyemids (Van Beneden, 1876). His belief was that this group occupied an evolutionary intermediate position between the Protozoa (unicellular animals) and the Metazoa (multicellular animals), and hence he created the intermediate name Mesozoa Van Beneden, 1876. Since then, numerous attempts have been made to classify the dicyemids, however, often leading to additional confusion rather than resolution. In particular, the dicyemid mesozoans have been suggested to be members of the Spiralia (based on developmental studies and encoding of a 'spiralian peptide' in the dicyemid *DoxC* gene (Furuya et al., 1992a; Kobayashi et al., 1999), highly simplified bilaterians (based on tool-kit *Pax6* and *Zic* genes; Aruga et al., 2007), ancient multicellular animals (based on 5S rRNA gene; Ohama et al., 1984), relatives of nematodes (based on 18S rRNA sequences; Pawlowski et al., 1996), closely affiliated to annelids (based on 18S and 28S rRNA sequences; Petrov et al., 2010), and a sister group to the clade consisting of annelids and molluscs (amino acid sequences of innexin; Suzuki et al., 2010). Recently, the transcriptome of *Dicyema japonicum* was sequenced with the authors presenting support for the placement of Dicyemida with the Orthonectida in phylum Mesozoa, which then forms a sister group to the clade of Mollusca and Annelida (Lu et al., 2017). However, differences in internal features and stages of their respective life cycles still shed a level of doubt on the dicyemids and orthonectids grouping together within a single phylum. Further transcriptome sequence data from additional dicyemid and orthonectid species, including representatives from all of the described genera, will be required to validate these findings and confirm the definite position in the Tree of Life of the dicyemid mesozoans.

Collection and Staining Methods

As dicyemid mesozoans are minute and comprise only a few cells, they rapidly degrade following host death so collection should be targeted from fresh material. After euthanasia, place the body of the cephalopod ventral side up in a tray and open the mantle cavity with a sterile scalpel blade to expose the paired renal sacs. Using forceps and scissors sterilized in absolute ethanol to avoid cross contamination, remove the left and right renal appendages, and smear small pieces onto glass microscope slides. A smear is made by holding the renal appendage between the forceps in one hand and the glass slide in the other, and then with slight pressure, moving the renal appendage across the glass slide from left to right covering the slide surface from the top to the bottom in straight parallel lines. If the host is infected by dicyemids, typically small white strands will be seen on the glass slide when it is held up to the light. At a minimum, 4 smears should be made per renal appendage (8 smears per host), although the number of smears can be increased for larger host individuals. Smears will then need to be fixed immediately in 70% ethanol to avoid parasite desiccation, with Lock-Mailer™ jars (Ted Pella, Inc.) proving to be ideal for field sampling and storage before slides are stained and mounted upon returning to the laboratory.

Although a range of staining methods have been used by past authors, a trial performed by Catalano et al. (2014) suggests staining with Ehrlich's acid haematoxylin diluted 20 parts of MilliQ water to 1 part stain for 20 minutes, dehydration in an ethanol series and counterstaining in eosin (70% ethanol for 10 minutes, 90% ethanol for 10 minutes, eosin 1% alcoholic solution diluted 20 parts of MilliQ water to 1 part stain for 2 minutes, and 100% ethanol for 15–20 minutes) as ideal for visualization of distinguishing morphological characters. Stained smears can then be mounted in Canada balsam, dried on a hot plate at 50 °C and examined with a compound microscope at magnification up to × 1,500, with drawings and measurements made with the aid of an ocular micrometer and drawing tube.

To confirm host species identification, it is desirable to collect a section of the host tissue (for example, mantle tissue) in 100% DNA-grade ethanol for molecular analysis. A section of each renal appendage can also be collected and stored in 100% DNA-grade ethanol for molecular analysis of the dicyemid parasite to complement traditional morphological classification. Depositing stained slides in registered museum collections should be mandatory for all new dicyemid species descriptions.

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Part V: ECTOPARASITES

Preface

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IMPETUS FOR PREPARING THIS BOOK

The United Nations (UN) has declared education as a basic human right. One of the UN's sustainable development goals is a call to ensure "inclusive and equitable quality education and promotion of lifelong learning opportunities for all" (United Nations, 2023; see also WOERC, 2012). Depending on the specifics of their implementation, financing, and dissemination models, open educational resources (OERs) have the potential to help in the effort to achieve equitable learning across the globe (Orr et al., 2015; Lee and Lee, 2021; see also Bali et al., 2020).

Open educational resources are "teaching, learning, and research materials in any medium that reside in the public domain or have been released under an open license that permits their free use and re-purposing by others" (Creative Commons, 2014). Wiley (2020) cites the Creative Commons' framing of OERs as providing explicit permission to "retain, re-use, revise, remix, and redistribute" openly-accessible educational material.

Aside from the obvious benefit of saving students money, OERs have been shown to promote equity among students. Their use has been shown to contribute to maintenance or improvement of student success, especially with respect to retention in school, course completion, grade point average, and subsequent educational attainment (Colvard et al., 2018; Griffiths et al., 2022; Fischer et al., 2015).

HOW TO USE THIS BOOK

Scope

This is a textbook covering concepts in animal parasitology. It is meant to be used by students, teachers, professors, researchers, and members of the public who are interested in learning about animal parasite biology, systematics, taxonomy, zoogeography, and ecology. The primary intended audience is upper-level undergraduate or graduate university students who have knowledge of basic biology and, particularly, basic animal biology.

Organization of the Book

This textbook was conceived to fill a gap in educational materials about parasitology. One of the main goals in both teaching and learning about parasites and parasitology is to understand the diversity of parasites and of parasitism as a way of life on Earth. With this in mind, the editors made a decision to treat the organization of the book as though led by the organisms themselves—a sort of bottom-up approach—and present the parasitic organisms as a parasitologist will first find them in nature, as in: Where they tend to exist in relation to their host, and more specifically, whether inside or outside the host animal. Therefore, the book includes sections covering a few taxonomic groups representing just some of the millions of extant endoparasite (Greek: **endo** = inside; **para** = beside; **sitos** = food) and ectoparasite (Greek: **ektos** = outside) species.

Examples of endoparasites are parasitic trematodes or nematodes that live inside the respiratory systems or gastrointestinal tracts of their hosts. Ectoparasites include lice and ticks, almost all fleas, many mites, a few platyhelminths that live on echinoderms, and even some chordates like the lamprey and vampire bat. Some groups of animals, such as monogeneans and mites, are not neatly categorized and may live part of their lives as endoparasites and part of their lives as ectoparasites or as free-living animals. Despite these myriad variations, the editors believe that the basic division between endo- and ecto- serves well enough to organize the chapters.

In approaching the organization in this way, the focus of the book is primarily at the level of species and other lower level taxonomy as opposed to higher-level groupings which are notoriously constantly in flux. The classification of parasites based on phylogenies is useful and necessary to understand the diversity, diversification, and evolution of parasites, but classification does not dictate the book's primary organization. Instead, the concept of biodiversity of parasites and their animal hosts is the main factor that motivates the research and teaching in the Harold W. Manter Laboratory of Parasitology (University of Nebraska State Museum, Lincoln, Nebraska, United States) where editor Scott L. Gardner conducts his work. It is this push toward understanding biological diversity of parasites that overarchingly informs the organization of this book.

Note about Bibliographical References

The citations in the book are formatted to promote finding usable copies, they are not meant to serve as an archival resource. As such, and to save space, only the first four authors are listed for each resource. A digital object identifier (doi) is included whenever one could be found; but the dois are not

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Accompanying Glossary

A supplemental glossary is in the process of preparation. Until the glossary is completed, a work that may be used in its stead for many of the terms found in the book is the Dictionary of Invertebrate Zoology (Maggenti et al., 2017) available online for free: https://digitalcommons.unl.edu/zeabook/61/

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Although students of pre-medical studies, medical studies, or veterinary studies may use this text to learn foundational concepts in animal parasitology, it is not a medical or veterinary text. Further, it is not meant for any medical- or veterinary-related purposes whatsoever. When medical or veterinary topics are touched upon in the text, this is for educational purposes for those studying or interested in the biological sciences generally. *No medical or veterinary advice of any kind is offered or implied anywhere in this textbook*. *No* medical or veterinary diagnoses, treatments, or conclusions of any kind may be construed using the knowledge offered herein.

For studies specifically related to medical parasitology, readers may consult any of a number of qualified texts in the subject, including Medical Parasitology: A Textbook (Mahmud et al., 2017), Medical Parasitology (Satoskar, 2009), and Modern Parasitology: A Textbook of Parasitology, 2nd edition, (Cox et al., 2009), among others. Numerous medical periodicals are also appropriate sources of knowledge about medical parasitology. For medical diagnoses, qualified practitioners of medicine may be consulted directly.

For studies specifically related to veterinary parasitology, readers may consult any of a number of qualified texts in the subject, including Veterinary Parasitology, 4th edition, (Taylor et al., 2015) and Georgis' Parasitology for Veterinarians, 11th edition, (Bowman, 2020), among others. Numerous veterinary parasitology periodicals are also appropriate sources of knowledge about veterinary parasitology. For veterinary diagnoses, qualified practitioners of veterinary medicine may be consulted directly.

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Invitation to Review and Give Feedback

If any qualified readers would like to serve as a reviewer for any of the sections, you are invited to please contact one of the editors to discuss the possibility of being assigned the task of reviewing. You will be credited in revisions if you ultimately serve as a selected reviewer. In addition, if readers discover factual or typographical errors in the content, please contact one of the editors.

HOW THE BOOK WAS DEVELOPED

Origin of the Book

The concept for this book arose in 2018 around the time there was a concerted push to create open educational resources in universities (Austin, 2018; Sennott et al., 2015). This push seemed well-timed to the editors. In fact, the rising costs of textbooks has become a major problem for students to the point where it is basically untenable to expect students to pay for them anymore. The editors reasoned that it would be a good time to call on their esteemed and accomplished colleagues in academia to help create a new textbook in a massively collaborative endeavor, if they were willing to participate.

Also driving the idea of a new textbook, the seminal English-language parasitology textbook of our time, Gerald R. Schmidt and Larry S. Roberts' Foundations of Parasitology, 9th edition (Roberts et al., 2012), has recently gone out of print and there are no plans to update it. John J. Janovy, Jr., the lead author of the last several editions of the Schmidt and Roberts book, agreed that the creation of a new textbook was a good and timely idea.

Contributing to the decision to attempt the creation of a large-scale textbook project was the public access/open access platform available to the editors, namely, the Zea Books imprint of the University of Nebraska–Lincoln Libraries. In line with the OER ethos driving the creation of the content, this publishing imprint operates under a diamond open access model, such that neither the authors nor the readers have to pay to publish nor to read any work published as a Zea Book.

Development of the Book

At the time of the conception of the book idea, the edi-tors capitalized on the availability of visiting scholars in the Harold W. Manter Laboratory of Parasitology (Lincoln, Ne-braska, United States)—Griselda Pulido-Flores, Scott Monks, and Donald Gettinger, as well as local colleagues John J. Janovy, Jr. and Gabor Rácz, and student-colleagues Auggie Tsogtsaikhan Dursahinhan and Guin Drabik—and called to-gether a couple of meetings to discuss their idea with the group. They asked them to envision what they would like to see in a new textbook, one that would be available online for anyone with a computer connection to access for free. Among many other good ideas they shared, they suggested that the book could possibly include numerous links to other sources and interactive modules, and pointed out that the information may be kept more current than was possible with a printed volume. Colleagues Paul Royster, Linnea Fredrickson, Catherine Fraser Riehle, and Mary Bolin in the University of Nebraska–Lincoln Libraries (Lincoln, Nebraska, United States) also provided encouragement and expertise that helped the project on its way.

When preparing to solicit manuscripts for this project, based on the preliminary conversations with colleagues, the editors first prepared an outline of the concepts desired to have covered and then created streamlined style requirements (the instructions for authors and references style guide are available online here: https://digitalcommons.unl.edu/parasittext/). They then asked numerous colleagues—all experts in their subareas of parasitology—to contribute one or more sections based on the outline. So many of them agreed to write sections that it seemed that it really might be possible to create a high-quality work with the input of so many fine experts. Every one of them submitted manuscripts quickly.

The editors gave the authors quite a bit of latitude regarding how to approach their assignment to write sections. They provided an optional template to work from (available here), but use of this format was optional. They wanted the authors to be able to express themselves in the way they each felt was best to demonstrate knowledge of their respective areas of interest within the larger subject of animal parasitology. This liberal approach naturally resulted in some variation in presentation styles, which is perhaps a plus for the reader. It breaks up the tone and emphases from section to section, and the reader gets a sense of each author's different voice and approach. The editors have worked to retain much of each author's preferred style of presentation, but with normalizing of typography and other style elements to help the manuscript finally cohere as a unified whole.

Some of the sections were sent out for review. This review process was open, so the authors knew who was reviewing their work and the reviewers were aware that the authors knew they were reviewing. Reviewed sections are marked as such with the reviewer's name and affiliation. Whether reviewed or not, all of the sections were editor-reviewed by both editors: Sue Ann Gardner edited primarily for bibliographic details and style elements, and Scott L. Gardner edited primarily for content.

Delayed Publication

With best-laid plans, the editors started to review and edit the sections as soon as they were submitted. Then a great number of both quite-dire and less-dire issues arose that interfered with the ability to complete the editing and production in as timely a manner as intended (selected challenges include: The SARS-CoV-2 pandemic requiring remote teaching, a computer crash, a death in the family that then required weeks away from work and home, radical changes in administrations at the university, and other issues). With those issues finally

receding in impact, five years after the project began, the book will be published at long last.

Demographic Data About the Authors

With editor Scott L. Gardner's large network of expert parasitologist colleagues, it was possible to seek out scholars who are experts in their field. While the first consideration when deciding who to invite to participate was expertise, the editors further worked toward the desired goal of equity and inclusion in the selection of authors. One result was a 1:2 ratio of women to men. While this does not represent parity, it is an improvement over days past when the majority of authors would likely have been men. Another result of efforts at equity and inclusion was the participation of many au-thors from outside the United States. Approximately 40% of authors are US-American and the remaining 60% are from one of 14 other countries (Argentina, Brazil, Australia, Japan, Mongolia, Bulgaria, Czechia, Germany, Hungary, Norway, Russia, Spain, Mexico, or Canada). Almost half of the authors (44%) do not have English as their first language.

Spanish-Language Version

In late 2018, the Office of the President at the University of Nebraska–Lincoln (Lincoln, Nebraska, United States) issued a call for proposals for Inclusive Excellence Development at the university. The editors were awarded funds to go toward translation of the textbook. With this, the editors partnered with a local professor of Spanish-language translation, Yoanna Esquivel Greenwood, who has created Spanish-language versions for numerous chapters in the book. Thanks to her work, and perhaps with the added input of some of the Spanish speakers among the authors, a comprehensive Spanish-language translation is forthcoming.

Acknowledgement of Authors' Contributions

From the Editors, Scott L. Gardner and Sue Ann Gardner

We sincerely thank all of the authors of this collaborative work. Your excellent contributions and dedication to the ad-vancement of knowledge of animal parasitology have the po-tential to positively change the lives of countless students and teachers worldwide.

While we were grappling with challenges and distractions that delayed the editing of the manuscript of this book, we lost a few of our esteemed author colleagues. We wish to posthumously acknowledge Bernie Fried, Akira Ito, and Robin M. Overstreet for what turned out to be some of their truly late-career contributions. We miss them, and we feel so fortunate to have benefitted from their long-acquired knowledge and their willingness to join in on this project.

Dedication

From the Editors, Scott L. Gardner and Sue Ann Gardner

This book is dedicated to **all** of our academic forebears and mentors who made this effort possible—some of whom are authors* of sections of the book! We can't list everyone, but we can provide a truncated list to commemorate some people especially.

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