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Review

Development of Monoxenous Trypanosomatids and Phytomonads in Insects

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In this review, we summarize the current data on development of monoxenous trypanosomatids and phytomonads in various insects. Of these, Diptera and Hemiptera are the main host groups, and, consequently, most available information concerns their parasites. Within the insect body, the midgut and hindgut are the predominant colonization sites; in addition, some trypanosomatids can invade the foregut, Malpighian tubules, hemolymph, and/or salivary glands. Differences in the intestinal structure and biology of the host determine the variety of parasites' developmental and transmission strategies. Meanwhile, similar mechanisms are used by unrelated trypanosomatids, reflecting the limited range of options to achieve the same goal.

Highlights

Complete trypanosomatid life cycles have been studied for a handful of species, mainly those of medical importance (i.e., *Leishmania* and *Trypanosoma*).

The vast majority of trypanosomatids infect insects of the orders Diptera and Hemiptera, although representatives of at least three additional orders have been implicated as specific hosts.

While monoxenous trypanosomatids develop mainly in the insect intestinal tract, some of them also live in Malpighian tubules, hemolymph, and salivary glands.

Developmental strategies of trypanosomatids in insects depend on the organization of the digestive system, feeding habits, and life cycles of their hosts.

Overview of Trypanosomatid Lifestyles

Trypanosomatids (Euglenozoa: Kinetoplastea: Trypanosomatidae) are obligate parasitic flagellates whose evolution was mainly shaped by adaptation to various animal hosts [1]. There are two notable exceptions: the genus *Phytomonas* adapted to vascular plants with transmission by phytophagous bugs [2,3], and a few species (at least one of which belongs to the genus *Herpetomonas*) switched to parasitism in the macronuclei of ciliates [4]. Traditionally, trypanosomatids are united into two nontaxonomic groups based on the type of life cycle: monoxenous flagellates undergo complete development in a single host individual (predominantly an insect), while dixenous ones require two distinct hosts, one of which functions as a vector and is typically an insect, while another is a vertebrate or a plant [5]. Phylogenic inferences convincingly demonstrated three independent transitions to dixeny in Trypanosomatidae [6].

Despite the apparent differences, the border between the two aforementioned groups is not impenetrable. Secondary transitions from dixeny to monoxeny have been described in *Phytomonas nordicus*, a parasite of predatory bugs, which does not develop in plants [7], and *Trypanosoma brucei equiperdum*, which completely switched to direct transmission between the vertebrate hosts [8]. Meanwhile, a number of monoxenous trypanosomatids have been recognized or suspected as agents of opportunistic infections in plants [9,10] and vertebrates, including humans [11,12].

Currently the family Trypanosomatidae unites 24 genera, 19 of which are monoxenous (Figure 1). The present classification system is still far from perfect and does not reflect the true diversity of the group: some genera (*Crithidia* and *Leptomonas*) are not monophyletic, some (*Borovskyia*, *Lafontella*, *Lotmaria*, *Kentomonas, Novymonas*, *Paratrypanosoma*, *Sergeia*) contain only a single described species, whereas several unnamed lineages are known only by sequences [13]. In addition, several trypanosomatid genera, described in the past, have not been reisolated and analyzed with molecular methods thus far. All these factors render any generalization preliminary at this point. However, the growing gap between the data from the 'premolecular era' and those obtained with rapidly developing modern techniques must be addressed before it becomes too wide. Since the life cycles of *Leishmania* and *Trypanosoma* spp. have been fairly well studied, at

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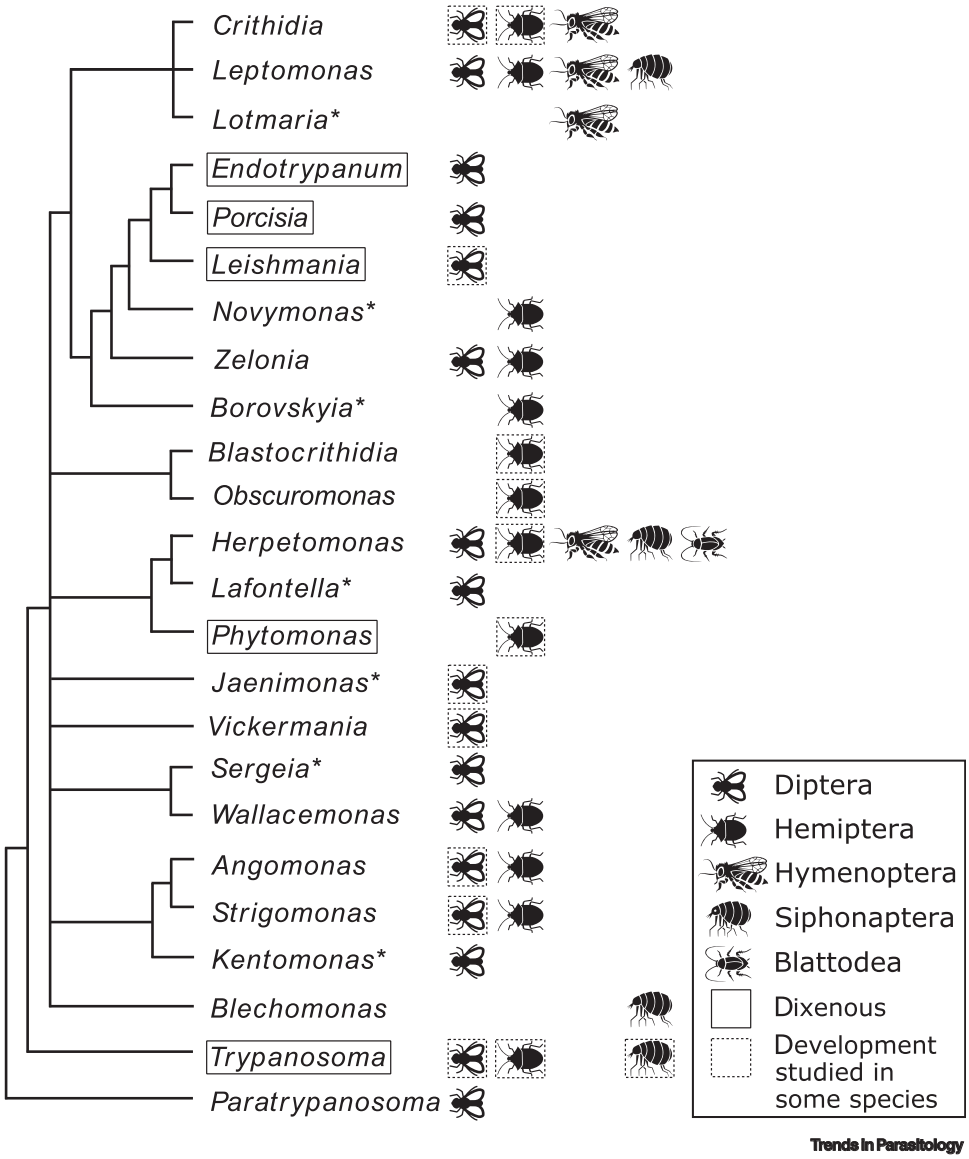

Figure 1. Schematic Phylogenetic Tree of Trypanosomatidae Based on the 18S rRNA Sequences. Recorded insect hosts are indicated for each genus; dixenous genera are marked by boxes; genera with a single described species are indicated by an asterisk. The tree is based on the data summarized in [13].

least for the most important pathogens from these groups (Box 1), we focused this review on monoxenous trypanosomatids and phytomonads (*Phytomonas* spp.).

Insect Hosts of Monoxenous Trypanosomatids and Phytomonads

Exceptional evolutionary plasticity of insects allowed them to colonize most ecological niches, where they usually dominate, in terms of both diversity and biomass [14]. Consequently, parasites of insects can be as diverse and successful as their hosts, and trypanosomatids are a good example of such a case. Regardless of whether these flagellates are transmitted via the external environment (monoxenous species) or directly between two different hosts (dixenous species), with just a few exceptions, their infective stages can pass through different insects. However,

Glossary

Amastigote: the classical amastigote is the same as the **endomastigote**, whereas the **cyst-like amastigote** is a cell that does not have any flagellum at all.
Brush border: the surface of an epithelium covered by tightly spaced microvilli, giving it the appearance of a fuzzy fringe.
Commensals: symbionts with no positive or negative impact on their hosts.
Constricted region: a segment of the midgut with an extremely narrow lumen filled with hypertrophied and tightly packed microvilli (present in some phytophagous bugs).
Cyst-like amastigotes: cells with no flagellum and flagellar pocket, no specialized outer envelope (in contrast to true cysts), with very condensed cytoplasm and a thick protective submembrane fine-grain layer.
Endomastigotes: cells of various shapes with a flagellum not exceeding the margins of the flagellar pocket or only slightly protruding from it. This term is used for monoxenous trypanosomatids and phytomonads, whereas the less precise term 'amastigote' is historically used for *Trypanosoma* and *Leishmania* spp.
Epimastigote: a cell with the flagellum exiting laterally and attached to the cell body; the kinetoplast is situated anteriorly to the nucleus.
Facultative host: a host which is not necessary for parasite circulation and which is unable to support it in the absence of obligate hosts.
Filopodia: filiform, sometimes branching, projections of the cellular membrane; among the trypanosomatids they are known in *Trypanosoma* and *Blastocrithidia* spp. and are 50–70 µm in diameter.
Hemidesmosome: a multiprotein complex ensuring stable cell adhesion on various surfaces; it is visible on transmission electron microscopy pictures as dark plaques associated with the cell membrane.
M4B: in some phytophagous bugs, a segment of the midgut following the constricted region and filled with a very viscous secretion.
Micropopulation: a monospecific group of individuals restricted to a small homogeneous area (in the case of parasites, to one host or its particular part).

Box 1. Overview of Life Cycles in *Trypanosoma* and *Leishmania* spp.

Trypanosoma cruzi

Triatomine bugs feeding on infected mammals obtain **trypomastigotes** and **amastigotes** with blood. In the bug midgut, trypomastigotes transform to amastigotes, which proliferate and transform to **epimastigotes**. The latter also proliferate, and some of them reach the midgut brush border, attaching to it with their unmodified flagellum. Eventually, they appear in the rectum, attaching to the rectal pads' cuticle and transform to metacyclic (infective, nondividing) trypomastigotes, which are discharged with feces. They cause infection in a mammal when getting onto a mucosal surface or into a wound caused by a bug's bite [99].

Salivarian *Trypanosoma* spp.

During bloodsucking, tsetse flies acquire bloodstream-form trypomastigotes, which transform into replicative procyclic ones in the midgut (e.g., *T. brucei*, *T. congolense*). After migration anteriorly through proventriculus to the salivary glands (*T. brucei*), or to the proboscis and hypopharynx (*T. congolense*), they attach via the lateral flagellar surface to the epithelium or to the cuticular lining, respectively, and multiply as epimastigotes. Finally, they transform into metacyclic trypomastigotes, which are injected with saliva during blood feeding on a mammal [100]. *Trypanosoma vivax* completes its development in the mouthparts and does not have midgut stage.

Trypanosoma rangeli

Trypomastigotes from mammalian blood get into the midgut of a triatomine bug, transform to epimastigotes, and multiply. Epimastigotes disrupt intestinal wall and migrate to the hemolymph, then traverse the salivary glands' epithelium in vacuoles. Within the glands, they attach to epithelial brush border, divide, and eventually transform to free-swimming metacyclic trypomastigotes [101].

Trypanosoma lewisi

When feeding on infected rats, fleas ingest trypomastigotes. These invade epitheliocytes of the midgut and proliferate inside parasitophorous vacuoles. After disruption of the host cells, trypomastigotes are released and either invade new epitheliocytes or migrate to the rectum to be discharged with feces [102].

***Leishmania* spp.**

Sandflies feeding on infected vertebrates ingest macrophages with amastigotes. The latter transform into procyclic promastigotes, multiplying inside the bloodmeal surrounded by peritrophic matrix, which starts decaying at the posterior end when digestion completes. Then promastigotes become more active, escape to ectoperitrophic space, attach to the midgut epithelium by inserting their flagella between microvilli, and proliferate. Later, they migrate to the anterior midgut, where some of them attach with extended flagellar tip to the stomodeal valve cuticle and destroy it with chitinase. Others produce proteophosphoglycan gel, which encloses vertebrate-infective metacyclic promastigotes and is regurgitated during sandfly feeding into the bloodstream of a vertebrate, causing infection [55]. A similar mechanism has been proposed to exist in mosquito-transmitted avian trypanosomes [103].

Microvilli (*singular*: microvillus): microscopic projections of the cellular membrane increasing the surface of a cell in order to enhance absorption, secretion, and other processes.
Nonspecific host: a host in which regular parasite development is impossible and infection is transient.
Obligate host: a host that is necessary for parasite circulation and able to support it alone (in monoxenous life cycles) or together with the complementary hosts (in di- and tri-xenous life cycles).
Promastigote: an elongated trypanosomatid cell with apically exiting flagellum and kinetoplast situated anteriorly to the nucleus.
Specific host: a host in which the parasite can undergo regular development, ensuring its transmission to the next host.
Transovum transmission: ingestion of infective parasite stages by newly hatched nymphs from the surface of their eggs with mother's feces bearing obligate intestinal bacterial endosymbionts.
Trypomastigote: a cell with the flagellum exiting laterally and attached to the cell body; the kinetoplast is situated posteriorly to the nucleus.
Typing unit: a group of organisms with identical or very similar (up to a certain threshold) sequences of a marker gene (e.g., SSU rRNA); typing units are regarded as proxies of species in molecular diversity studies.

the parasite development is possible only in **specific hosts** (see Glossary). Specificity, in this case, is a result of the action of coevolutionary forces upon a particular trypanosomatid species and its host [15]. Experimental confirmations are required to assess this reliably, but only several studies have implemented such an approach so far. Thus, *Crithidia bombi*, isolated from the bumblebee *Bombus lucorum*, and *C. mellificae* from the honeybee *Apis mellifera*, can infect bees from other families, while cross-infections were unsuccessful [16]. Interestingly, another honeybee parasite, *Lotmaria passim*, is strictly confined to one host, *A. mellifera* [17]. A dipteran parasite *Jaenimonas drosophilae*, inhabiting *Drosophila falleni*, can infect larvae of other *Drosophila* spp. and successfully overcome host metamorphosis [18]. Experimental infections of blow flies (Calliphoridae) have shown that the flagellate *Vickermania spadyakhi*, isolated from the ensign fly *Nemopoda nitidula* (Sepsidae), can successfully develop and be horizontally transmitted between individuals of *Lucilia sericata*, but merely survives in *Calliphora vicina* [19]. The aforementioned examples cannot draw a comprehensive picture but suggest that monoxenous trypanosomatids do not have a universal strategy in respect to specificity. Assessing the latter is further complicated by the tendency of these flagellates to infect **nonspecific hosts** and **facultative hosts**. An example of a nonspecific host is *A. mellifera*, serving as a carrier of *C. bombi* infective for bumblebees [20]. For the secondarily monoxenous trypanosomatid

P. nordicus, developing in the predatory shield bugs *Troilus luridus* and *Picromerus bidens* [7,21], the former, hibernating at imaginal stage with parasites inside the body, is the **obligate host**, while the latter, overwintering as a parasite-free egg, is facultative. Of note, in most other described phytomonads, insect hosts (vectors) are unknown, making it impossible to judge their specificity.

An alternative approach to assess specificity is analyzing data on the parasite prevalence in populations of a given host [22]. While specificity and occurrence are not the same, high parasite prevalence in a certain host species may indicate the specific nature of their relationship. However, it is necessary to take into account the potential issues and pitfalls, such as limited number of analyzed specimens or predatory nature of the hosts, which may lead to high rates of nonspecific infections [23,24].

Although the number of nominal trypanosomatid genera has almost doubled over the past two decades [1], this had very little impact on the global picture of host preference by monoxenous trypanosomatids and phytomonads (Figure 1). Insect orders Diptera and Hemiptera still significantly prevail, followed by Hymenoptera, Siphonaptera [22], and Blattodea recently added to this list [25]. It remains to be confirmed whether Lepidoptera, Trichoptera, Mecoptera, and Phthiraptera, in which trypanosomatids were previously recorded [6], can host these parasites specifically.

Although most of the over 300 **typing units** (TUs) of monoxenous trypanosomatids and phytomonads, identified in biodiversity assays, are parasites of Hemiptera, this reflects rather an experimental bias than the real host preference since most of these studies were focused on true bugs [6]. Hemipterans were recorded as hosts of 13 trypanosomatid genera (Figure 1), but only cyst-forming monoxenous trypanosomatids (*Blastocrithidia* and *Obscuromonas*) and dixenous *Phytomonas* appear specific to this order of insects. In addition, *Borovskyia barvae* and the symbiont-containing *Novymonas esmeraldas* are the single members of their genera known only from herbivorous bugs of the families Miridae and Rhopalidae, respectively [26–28].

Flagellates of the genera *Angomonas*, *Crithidia*, *Herpetomonas*, *Leptomonas*, *Strigomonas*, *Wallacemonas*, and *Zelonia* were documented in both Diptera and Hemiptera (Figure 1). It was proposed that these two host groups may exchange parasites either via predation or copro-/necrophagy with the transient acquisition of a dipteran parasite by a predatory bug seeming more likely [6,29,30]. However, a widespread Palearctic species *Crithidia brevicula* appears to be a generalist capable of infecting bugs of the families Nabidae, Gerridae, and (nonpredatory) Miridae [31], as well as various dipterans: mosquitoes *Culex* spp. and flies of the families Calliphoridae, Muscidae, Heleomyzidae, Sepsidae, and Antomyidae [32,33]. Out of the 13 monoxenous trypanosomatid genera, documented in Diptera (over 50 TUs), 6 were documented exclusively in these insects: *Jaenimonas*, *Kentomonas*, *Lafontella*, *Paratrypanosoma*, *Sergeia*, and *Vickermania* (Figure 1, [13]). Collectively, monoxenous trypanosomatids of Diptera and Hemiptera account for over 90% of the recognized family diversity [6]. In Hymenoptera, trypanosomatids of four genera (*Crithidia*, *Herpetomonas*, *Leptomonas*, and *Lotmaria*) have been detected. Only the monotypic genus *Lotmaria* is exclusive to these insects [17]. Siphonaptera can be infected by *Blechomonas*, *Herpetomonas*, and *Leptomonas*, but only *Blechomonas* is specific to fleas [34]. Thus far, only *Herpetomonas* spp. were identified in Blattodea [25].

Dipteran insects most likely were the primary trypanosomatid hosts: (i) mosquito-infecting *Paratrypanosoma confusum* represents the earliest branch on the trypanosomatid phylogenetic

tree [35]; (ii) the most ancient trypanosomatids found to date were detected in mosquitoes from Early Cretaceous (~100 mya) amber [36,37]; (iii) most trypanosomatid genera have dipteran hosts. Adaptation of trypanosomatids to true bugs and other host groups then must have occurred via horizontal transfer.

Localization in Insects

As mentioned previously, life cycles have been described for only a few trypanosomatid species (Figure 1). Notably, for almost one-third of the monoxenous trypanosomatid genera, not only their life cycles but also their localization in the host are not known (Figure 2). By 'localization' of parasites we mean their ability to form stable, spatially isolated and proliferating **micropopulations** in certain host organs or tissues [38,39]. These micropopulations are not discrete, since localization of parasites may gradually change.

Monoxenous trypanosomatids parasitize the digestive system of insects, which is divided into anterior (foregut), middle (midgut), and posterior (hindgut) sections (Figure 2A). In the anterior and posterior parts, epithelium is covered with cuticles, while in the midgut, where the food is digested and the nutrients are absorbed, it bears a **brush border** of **microvilli** on the apical surface (also present on epitheliocytes of the Malpighian tubules and salivary glands). Various peritrophic structures formed in the midgut play an important role in the interactions with parasites [40].

Some trypanosomatids are confined to specific locations within the host. For example, *Lotmaria passim* and many *Crithidia* spp. develop in the rectal ampullae [17,41,42], while *J. drosophilae* and *Vickermania* spp. colonize the endoperitrophic space of the host midgut (Figure 2B) [18,19]. Conversely, many trypanosomatids develop in multiple places forming spatially separated micropopulations (Figure 2C) [7,43–45].

While monoxenous trypanosomatids develop mainly in the insect intestinal tract, some of them also inhabit glandular appendages of the host digestive system – Malpighian tubules or salivary glands (Figure 2D–G) [46–49]. While passing the digestive tract, some *Blastocrithidia* spp. can perforate the intestinal wall, settling under the basal lamina [44,50], or penetrate through it into the coelomic epithelium and further into the host hemolymph (Figure 2B), as *Leptomonas pyrrhocoris*, *Strigomonas culicis*, and *Phytomonas* spp. [7,51–53]. It is difficult to distinguish specific localization from temporary presence of parasites in a particular part of the host body without experimental studies. However, the active proliferation and presence of specific morphotypes in a particular organ or tissue can serve as evidence of specific localization.

Foregut

After being taken with food, most trypanosomatids pass the insect foregut without delay. The only known exception among monoxenous trypanosomatids is *Herpetomonas nabiculae*, colonizing mainly the pharyngeal valve of the predatory damsel bug *Nabis flavomarginatus.* These flagellates attach to the cuticle using dilated flagellar tips (Figure 3A) with **hemidesmosomes** and form a giant 'rosette'. Their well-developed cytostome–cytopharyngeal complex, which is quite atypical for monoxenous trypanosomatids, may play an important role in the adaptation to this habitat [54]. In contrast, the localization on the surface of the host's foregut cuticle is common for dixenous *Leishmania* spp. and avian trypanosomes [55,56].

Midgut

Many trypanosomatids specifically inhabit the midgut (Figure 2D–G), while others inevitably pass through it either to settle elsewhere inside the insect body or to be discharged with feces for

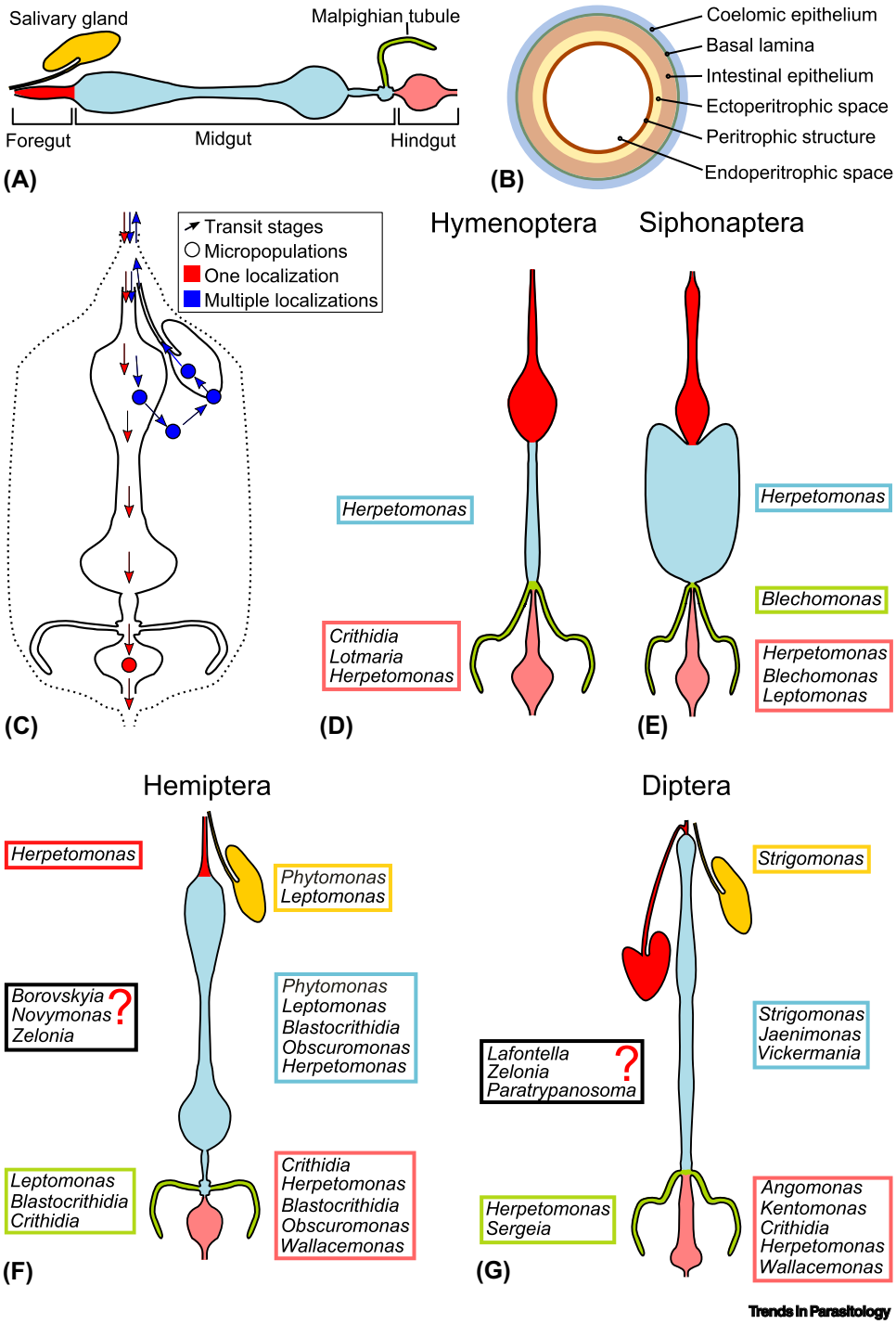

Figure 2. Localization of Trypanosomatids in Insects. (A) General scheme of insect digestive system. (B) Cross section of midgut. (C) Examples of developmental pathways with single and multiple localizations. (D–G) Localization of trypanosomatids in different insect orders; the question mark indicates that localization is unknown. The color scheme in panels (D–G) is the same as that in (A).

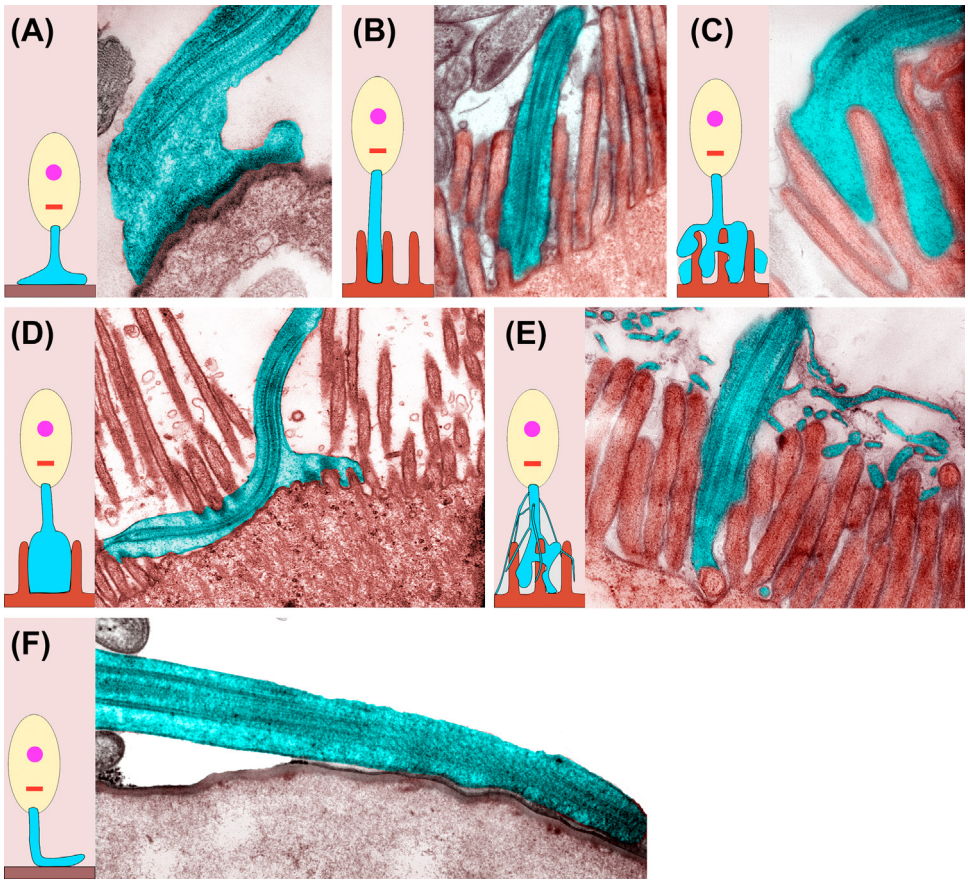

Figure 3. Modes of Trypanosomatid Attachment to the Epithelia of Digestive and Excretory Systems. (A) Attachment to cuticle by a dilated flagellar tip. (B) Flagellum entanglement among microvilli. (C) Attachment with comb-like flagellar projections embracing microvilli. (D) Attachment to the area with reduced microvilli using lateral flagellar surface. (E) Attachment to microvilli with filopodia-like projections. (F) Attachment to cuticle with lateral flagellar surface. Each panel contains a schematic diagram (left) and colored electron micrograph (right). Flagella are colored in cyan; cuticular and microvillar epithelia are in different shades of brown. Please note that *Trypanosoma* and *Leishmania* spp. are left out of the figure.

subsequent transmission. The diversity of adaptations to living in the midgut is associated with differences in its organization among insect taxa. The most important in this respect are the selectively permeable peritrophic structures, which form two compartments for successive stages of food digestion in the midgut (endo- and ecto-peritrophic spaces, Figure 2B) and protect the epithelium from the coarse food particles and pathogens [57]. In Hemiptera, these structures are represented by perimicrovillar membranes [40], which are noncontiguous and, therefore, many trypanosomatids, living in the midgut, can overcome this barrier and reach the intestinal wall. *Blastocrithidia* and *Obscuromonas* anchor on the epithelium using one of the following mechanisms: (i) flagellum entanglement among microvilli; (ii) microvilli reduction and attachment to the host cell membrane with the enlarged tip or lateral flagellar surface; or (iii) comb-like projections of the flagellar tip embracing microvilli (Figure 3B–D) [44,50,54,58–60]. *Leptomonas pyrrhocoris* and *Phytomonas* spp. cannot attach to epitheliocytes and, while some flagellates reside in the lumen, others pass through the midgut wall into the hemolymph [7,53,61]. Some *Blastocrithidia* spp. also

traverse the intestinal epithelium, but do not perforate the basal lamina and reside under it multiplying there [44,50].

In the phytophagous bugs of the superfamilies Lygaeoidea and Coreoidea, the intestine has a peculiar organization preventing its colonization by most trypanosomatids. The anterior midgut portion, responsible for digestion and absorption of nutrients, is isolated from the endosymbiont-containing posterior one by two specialized segments, **constricted region** and **M4B,** preventing penetration of most microorganisms, including pathogens and unwanted competitors of the bugs' obligate bacterial symbionts [62]. Trypanosomatids, living in such hosts, have evolved two different strategies, as exemplified by two parasites of *Coreus marginatus* – *Phytomonas lipae* (dixenous) and *Blastocrithidia raabei* (monoxenous). The former passes from the host midgut to hemolymph and then to salivary glands, whereas the latter fiercely breaks through the isolating segments into the hindgut [44,53].

In contrast to Hemiptera, peritrophic structures in Diptera are contiguous and, therefore, insuperable for trypanosomatids. They represent a jelly-like matrix with two or three layers built of glycosaminoglycans, glycoproteins, chitin, and structural proteins – peritrophins [40]. High concentrations of nutrients, released during digestion in the endoperitrophic space of the fly midgut, are quite favorable for trypanosomatids, but attacks of the host immune system and digestive enzymes make the conditions there adverse [63]. Moreover, in a typical case, the peritrophic membrane is continuously produced by the foregut (ventriculus) epithelium and shifted towards the posterior part of the intestine [40], making the attachment to it unreasonable. As of today, only a few monoxenous species have been documented to specifically live in such conditions. One of these is *J. drosophilae*, infecting *Drosophila* spp., but no details of its development are available [18]. *Herpetomonas ampelophylae,* another parasite of *Drosophila* spp. (not yet verified with molecular methods), multiplies in the endoperitrophic space but then enters the ectoperitrophic space, opening near the rectal valve, and attaches to the intestinal epithelium by weaving the flagellum between microvilli [64]. *Herpetomonas muscarum* can penetrate between the two layers of the peritrophic membrane in the housefly *Musca domestica*, apparently also after finding a bypass [65]. A distinct strategy is used by *Vickermania* spp. restricted to the endoperitrophic space of Caliphoridae and Sepsidae flies. These flagellates start growing a second flagellum right after cell division and, until the end of the next division, they preserve the contact between the new and old flagella, beating as a single unit. This allows them to maintain efficient motility throughout the cell cycle and, therefore, minimizes the risk of discharge with intestinal peristalsis [19].

Since in Nematocera the peritrophic membrane is temporarily formed in response to feeding, trypanosomatids infecting such insects can exit from them and continue development on the epithelial surface, as can be exemplified by *Leishmania* (Box 1). In the experimental infection of the mosquito *Aedes aegypti* by *Strigomonas culicis*, the parasites were found on the surface of the midgut epithelium, attached by their flagella to the microvilli. Prolonged infection led to the degradation of the attachment zone and release of flagellates into the host's hemocoel [52].

Hindgut

The insect hindgut is colonized by many trypanosomatid species, some of which occupy only this niche, whereas others use it as an additional localization site (Figure 2D–G). Here, flagellates are attached to the cuticle either by an expanded flagellar tip (similarly to *Herpetomonas nabiculae* in the foregut), or by lateral flagellar surface (Figure 3F) [66]. In true bugs, trypanosomatids predominantly inhabit the surface of rectal pads and form clusters, often arranged in several rows [44,54,58,67,68]. These insect organs play an important role in the absorption of water and amino acids during the final stages of digestion [69]. After a bug dies, the rectal pads are

the last site that parasites leave [70]. In Diptera, bearing papillae instead of pads in the rectum, localization of trypanosomatids is different. Thus, *H. samuelpessoai* in the housefly *M. domestica* colonizes not the rectal papillae but only the area around them [71]. The mechanisms, facilitating these interactions, are poorly understood, but in triatomine bugs they may be mediated by hydrophobic molecules on the surface of hindgut epicuticle [72].

Malpighian Tubules

The Malpighian tubules open at the border between the midgut and hindgut. As in the midgut, their epithelial surface bears numerous microvilli, but without peritrophic structures [69]. Several monoxenous trypanosomatid species parasitizing Hemiptera, Diptera, and Siphonaptera have been documented in this location (Figure 2E–G) [34,46,58,73–76]. In order to anchor on the epithelial brush border, *Blastocrithidia gerridis* in *Gerris lacustris*, and *B. papi* in *Pyrrhocoris apterus*, use long (50–70 nm) tubular extensions intertwining with each other, microvilli, and flagella of other individuals, thus creating large, attached parasite associates [48,54] (Figure 3E). These extensions are morphologically and functionally analogous to the **filopodia** of the bloodstream forms of African trypanosomes [77,78]. *Crithidia flexonema* and *L. pyrrhocoris* can traverse the Malpighian tubules' wall and form micropopulations under the basal lamina and/or in the coelomic epithelium of *Gerris odontogaster* and *P. apterus*, respectively [51,54,58]. At least in the latter species, this process may be associated with the subsequent parasites' migration into the hemolymph and salivary glands [49].

Hemolymph

Only a small number of trypanosomatid species have been described from the insect hemolymph. Moreover, some of these accounts may have reported accidental contamination of hemolymph during insect dissection [79]. *Phytomonas* spp. use hemolymph to advance the intestinal stages to the salivary glands of the host, yet, as a rule, they do not divide there [43,53,80]. Conversely, *L. pyrrhocoris* can multiply in the hemolymph of *P. apterus* before invading the salivary glands [49,51]. Among parasites of Diptera, localization in the hemolymph followed by penetration into salivary glands was observed for *Strigomonas culicis* in experimental infection of mosquitoes [45,52]. Hemolymph invasion may also be associated with transphasic transmission and/or persisting during the host diapause, as described for *Herpetomonas swainei* in the sawfly *Neodiprion swainei* (Hymenoptera: Tenthredinidae) [81].

Salivary Glands

In hemipterans, trypanosomatid infection of the hemolymph (most thoroughly studied in *Trypanosoma rangeli*) is often associated with penetration into salivary glands [49,82–84]. While the biological meaning of the salivary glands' invasion by monoxenous parasites (e.g., *L. pyrrhocoris* [49]) remains unclear, in dixenous *Phytomonas* spp. this is the life-cycle phase necessary for transmission to plants [43]. This was thoroughly investigated in *P. nordicus* and *P. serpens*. From the hemolymph, **promastigotes** penetrate into the epitheliocytes and myocytes of the outer envelope of salivary glands, migrate within individual parasitophorous vacuoles to the basal lamina and, after breaking the latter, invade the gland epithelium. *P. nordicus* accomplishes this, being localized exclusively to vacuoles in which it intensively multiplies, whereas *P. serpens* passes by all possible ways: within vacuoles or directly through cytoplasm and intercellular spaces. After reaching the gland lumen, both species start active cell division – either being attached to host microvilli (*P. nordicus*) or free (*P. serpens*) – and finally produce infective **endomastigotes** [7,85]. This part of development of *P. oxycareni* in *Oxycarenus lavaterae* (Lygaeidae) and *P. lipae* in *Coreus marginatus* (Coreidae) is similar to that of *P. serpens* [53,80]. Of note, transmission via saliva is typical for some *Trypanosoma* spp. (Box 1).

Dispersal Stages and Transmission of Parasites

Invariably, the development of trypanosomatids in insects ends with the formation of stages, which are infective for the next host. Several types of such dispersal stages can be distinguished based on how they are formed, resistance to various external factors, and morphology (Figure 4).

Nonspecialized Intestinal Stages

The survival of nonspecialized trypanosomatid cells discharged with feces, and their ability to infect new hosts, depends entirely on the environmental conditions. *Crithidia fasciculata*, continuously dividing in the rectum of mosquito larvae, ends up in water, where it can survive for up to 7 days. The flagellates are captured by new larvae along with other planktonic

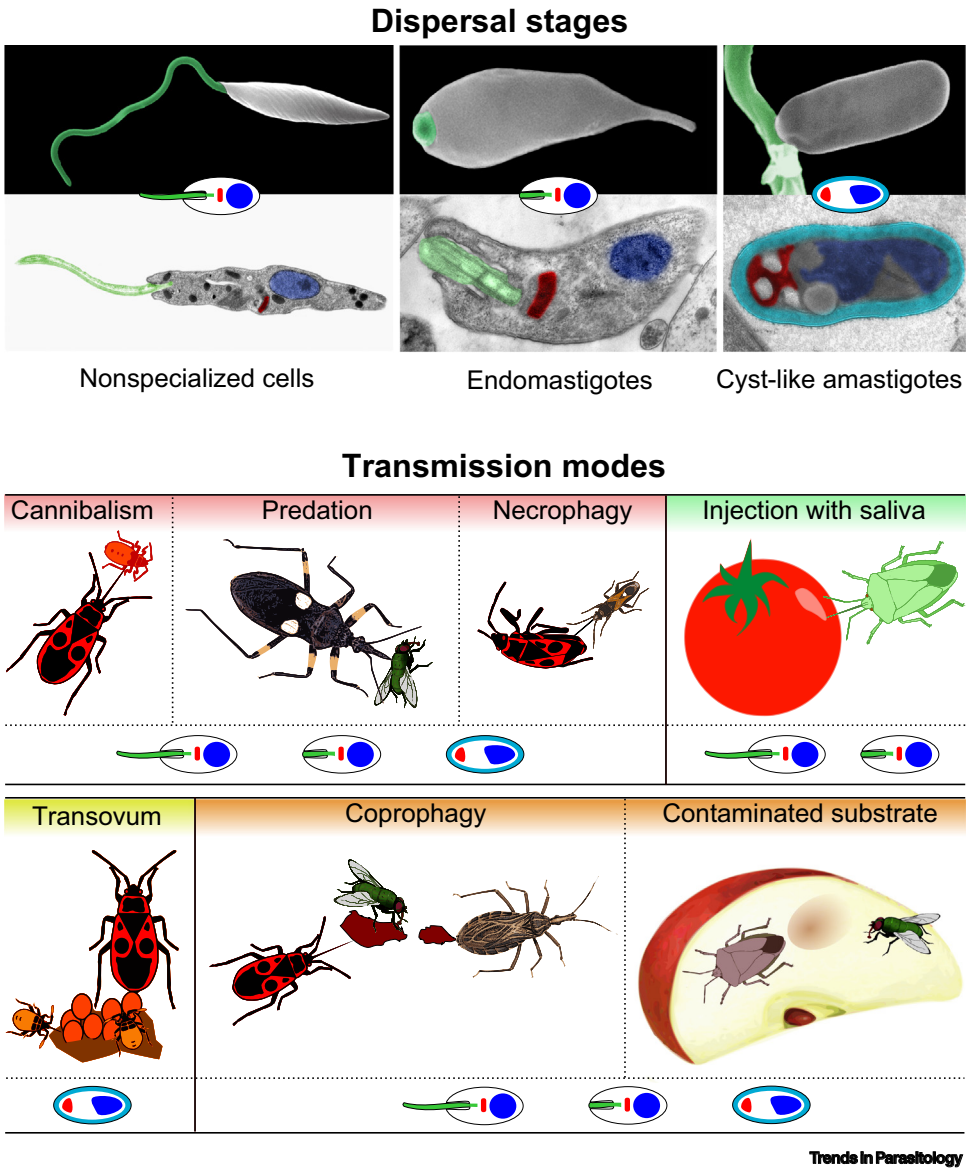

Figure 4. Dispersal Stages and Transmission Modes in Monoxenous Trypanosomatids and Phytomonads. Nucleus, kinetoplast, and flagellum are colored in blue, red, and green, respectively.

microorganisms and begin to reproduce again [86,87]. This type of transmission is widespread among monoxenous trypanosomatids infecting aquatic insects and those terrestrial ones whose development and/or feeding is associated with moist substrates, such as plant juices or decaying organic matter [9,18,19,88]. Nonspecialized intestinal stages can also be transmitted via predation, necrophagy, cannibalism, and coprophagy in those insects for which such behavior is typical [24,48,79].

Endomastigotes

Endomastigotes have been described in many monoxenous trypanosomatids and phytomonads, in which they are typically formed in the intestine and salivary glands, respectively. Interestingly, the secondarily monoxenous *P. nordicus* combines both variants [7], while in *L. pyrrhocoris* endomastigotes were documented in the host hemolymph and salivary glands [49]. This dispersal stage is morphologically indiscernible from amastigotes of *Leishmania* and *Trypanosoma* spp., although, to the best of our knowledge, they are functionally distinct (Box 1). Endomastigotes have been studied in more detail in *Crithidia brevicula*, which has two submicropopulations in the host rectum: free-swimming promastigotes, which occasionally form endomastigotes, and large attached spherical cells [41]. While the latter cells cannot withstand even short-term drying, endomastigotes remain viable in a dried drop of host feces or cultural medium for about 10 days. Upon cloning, these submicropopulations maintain their differences for up to 1 year and then the whole spectrum of forms reappears [41].

Cyst-Like Amastigotes

These dispersal stages, inherent to the phylogenetically related genera *Blastocrithidia* and *Obscuromonas* [89], have no analogs in other protists [90]. Typically, they are formed by budding, followed by binary divisions of daughter cells attached to the mother cell's flagellum, thus creating characteristic 'straphangers' [67,91,92]. **Cyst-like amastigotes** are extreme survivors. In dry feces of triatomine bugs, the virulence of *Blastocrithidia triatomae* was confirmed after 13 years [79]. Heating to 60°C, freezing in liquid nitrogen, and sonication of such cells of an unidentified cyst-forming trypanosomatid did not prevent infection of the bug *Oncopeltus varicolor* [93]. This incredible resistance allowed mastering a highly efficient mode of the vertical **transovum transmission**, exploiting the habit of the newly hatched bug nymphs to eat, from the egg surface, mother's feces containing obligate intestinal bacterial endosymbionts [59,94]. Complemented by more common horizontal modes, this ensures highly efficient transmission of cyst-forming trypanosomatids in the populations of their hosts. Massive cyst formation in *B. papi* is coordinated with the production of eggs by its firebug host [59].

Host–Parasite Interactions

Since the publication of the last review on host–parasite interactions over a quarter-century ago [79], the progress in this area remains limited. Most monoxenous trypanosomatids and phytomonads are historically considered harmless **commensals** [6]. However, many monoxenous species in various insect hosts were reported to cause increased mortality rates and decreased fitness, as judged by lowered efficiency of physiological processes and overall activity, as well as physical signs, such as body mass reduction and slow development [13]. The negative impact of these parasites on insects can involve competition for nutrients with the host and/or its obligate prokaryotic symbionts [95], blockade of the lumen (e.g., in Malpighian tubules) [48,96], and damage to tissues dealt as a result of attachment or migrations within the host body (see previous text).

Little is known about the host response to infections by monoxenous trypanosomatids and phytomonads. The presence of *J. drosophilae* in *D. melanogaster* larvae induces an immune response, including the production of antimicrobial peptides [18]. Some promastigotes of

Phytomonas spp., migrating through the host hemolymph, are immobilized by hemocytes [15,97]. Not only phytomonads, migrating through tissues, but also some (apparently persisting cells of) *Blastocrithidia* spp. reside and multiply within parasitophorous vacuoles [44,53], the molecular composition and functions of which are unknown in contrast to the well-studied genera *Trypanosoma* and *Leishmania* [98].

Concluding Remarks

Despite the scarcity of data on the development of trypanosomatids in insects, it is already possible to draw some conclusions. Although, in general, the life cycles of monoxenous trypanosomatids are simpler as compared to those in the dixenous ones, the diversity of their adaptations to insect hosts is comparable, while some of them are unique. It appears that strategies of inhabiting the midgut and hindguts of insects as well as colonization of their salivary glands might have evolved independently in different lineages of these flagellates as adaptations to particular hosts. Although, in many cases, the observed similarities are determined by limited options of implementing adaptations to similar conditions, sometimes they could also be explained by the common origin. It is not always easy to delineate these two scenarios, and we believe that developmental studies of monoxenous trypanosomatids, which are the closest relatives of dixenous species, will help to answer these questions (see Outstanding Questions).

Outstanding Questions

Why do most trypanosomatids infect Diptera and Hemiptera?

What determines wide or narrow host specificity in monoxenous trypanosomatids and phytomonads?

Why do monoxenous trypanosomatids occasionally invade salivary glands, while there is no known mechanism of transmission associated with it?

Can endomastigotes of monoxenous trypanosomatids proliferate in harsh environmental conditions, similarly to amastigotes of *Leishmania* and *Trypanosoma*?

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Declaration of Interests

The authors declare no competing interests.

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