Parasitology

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Farming, slaving and enslavement: histories of endosymbioses during kinetoplastid evolution

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Special Issue Review

Cite this article: Harmer J, Yurchenko V, Nenarokova A, Lukeš J, Ginger ML (2018). Farming, slaving and enslavement: histories of endosymbioses during kinetoplastid evolution. *Parasitology* **145**, 1311–1323. https://doi.org/10.1017/S0031182018000781

Received: 26 January 2018
Revised: 29 March 2018
Accepted: 8 April 2018
First published online: 13 June 2018

Key words:
Angomonas deanei; *Candidatus* Kinetoplastibacterium; cytostome; *Kentomonas*; *Novymonas esmeraldas*; *Pandoraea*

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Abstract

Parasitic trypanosomatids diverged from free-living kinetoplastid ancestors several hundred million years ago. These parasites are relatively well known, due in part to several unusual cell biological and molecular traits and in part to the significance of a few – pathogenic *Leishmania* and *Trypanosoma* species – as aetiological agents of serious neglected tropical diseases. However, the majority of trypanosomatid biodiversity is represented by osmotrophic monoxenous parasites of insects. In two lineages, novymonads and strigomonads, osmotrophic lifestyles are supported by cytoplasmic endosymbionts, providing hosts with macromolecular precursors and vitamins. Here we discuss the two independent origins of endosymbiosis within trypanosomatids and subsequently different evolutionary trajectories that see entrainment *vs* tolerance of symbiont cell divisions cycles within those of the host. With the potential to inform on the transition to obligate parasitism in the trypanosomatids, interest in the biology and ecology of free-living, phagotrophic kinetoplastids is beginning to enjoy a renaissance. Thus, we take the opportunity to additionally consider the wider relevance of endosymbiosis during kinetoplastid evolution, including the indulged lifestyle and reductive evolution of basal kinetoplastid *Perkinsela*.

Introduction

Kinetoplastids are one of three major groups of organisms that belong to the evolutionarily divergent protist phylum Euglenozoa (Cavalier-Smith, 2016). Although divergent, euglenozoans are ubiquitous; representatives from all three groups are easily isolated from many freshwater, marine and soil environments and, in part due to their overall abundance, contribute significantly to ecosystem ecology (von der Heyden *et al.*, 2004; Edgcomb *et al.*, 2011; Lukeš *et al.*, 2015; Mukherjee *et al.*, 2015; Flegontova *et al.*, 2016; Flegontova *et al.*, 2018).

Systematically, the kinetoplastids separate into the monophyletic, obligatory parasitic trypanosomatids and a wide diversity of free-living, bi-flagellate phagotrophs, with occasional examples of parasites and symbionts populating three major clades (von der Heyden *et al.*, 2004; Simpson *et al.*, 2006; Kaufer *et al.*, 2017; Yazaki *et al.*, 2017). It is the uniflagellate trypanosomatids that are the best known due to the role of some as the aetiological agents of serious, neglected tropical diseases (Nussbaum *et al.*, 2010). The defining characteristic common to both free-living and parasitic kinetoplastids is the coalescence (in trypanosomatids the catenation) of several thousand circular DNA molecules to form distinctive mitochondrial genome architectures, known more commonly as kinetoplasts, and which give rise to the class name Kinetoplastea (Lukeš *et al.*, 2002). Uridine-insertion and -deletion editing of mRNA on a massive scale is essential for gene expression from these genomes and provides a second example of extreme or unusual biology that defines and pervades throughout the kinetoplastids (Aphasizhev and Aphasizheva, 2014; David *et al.*, 2015; Read *et al.*, 2016). For further examples of extreme kinetoplastid biology that have peripheral relevance for this review – peroxisome-compartmentalized carbohydrate metabolism, loss of transcriptional control on protein-coding gene expression, flagellar pocket dynamics – readers are directed towards articles by Haanstra *et al.* (2016), Morales *et al.* (2016*a*), Clayton (2014), and Field and Carrington (2009).

Although trypanosomatid species are widely known as the causative agents for diseases of medical, veterinary and agricultural importance (Jaskowska *et al.*, 2015; Giordani *et al.*, 2016; Field *et al.*, 2017; Kaufer *et al.*, 2017), most members of the family are simply monoxenous parasites of insects (Podlipaev *et al.*, 2004; Maslov *et al.*, 2013; Lukeš *et al.*, 2014; Kaufer *et al.*, 2017) with not always a clear indication that these protists are pathogenic towards their invertebrate host(s). Also less widely recognized is that at least twice, the symbiosis between a bacterial endosymbiont and a host trypanosomatid has occurred (Du *et al.*, 1994; de Souza and Motta, 1999; Votýpka *et al.*, 2014; Kostygov *et al.*, 2016) (Fig. 1). Trypanosomatid taxa involved in these events are not particularly closely related, and different evolutionary trajectories are possibly evident for each symbiosis: in the Strigomonadinae, growth and division of a single bacterial endosymbiont is entrained within the cell cycle of

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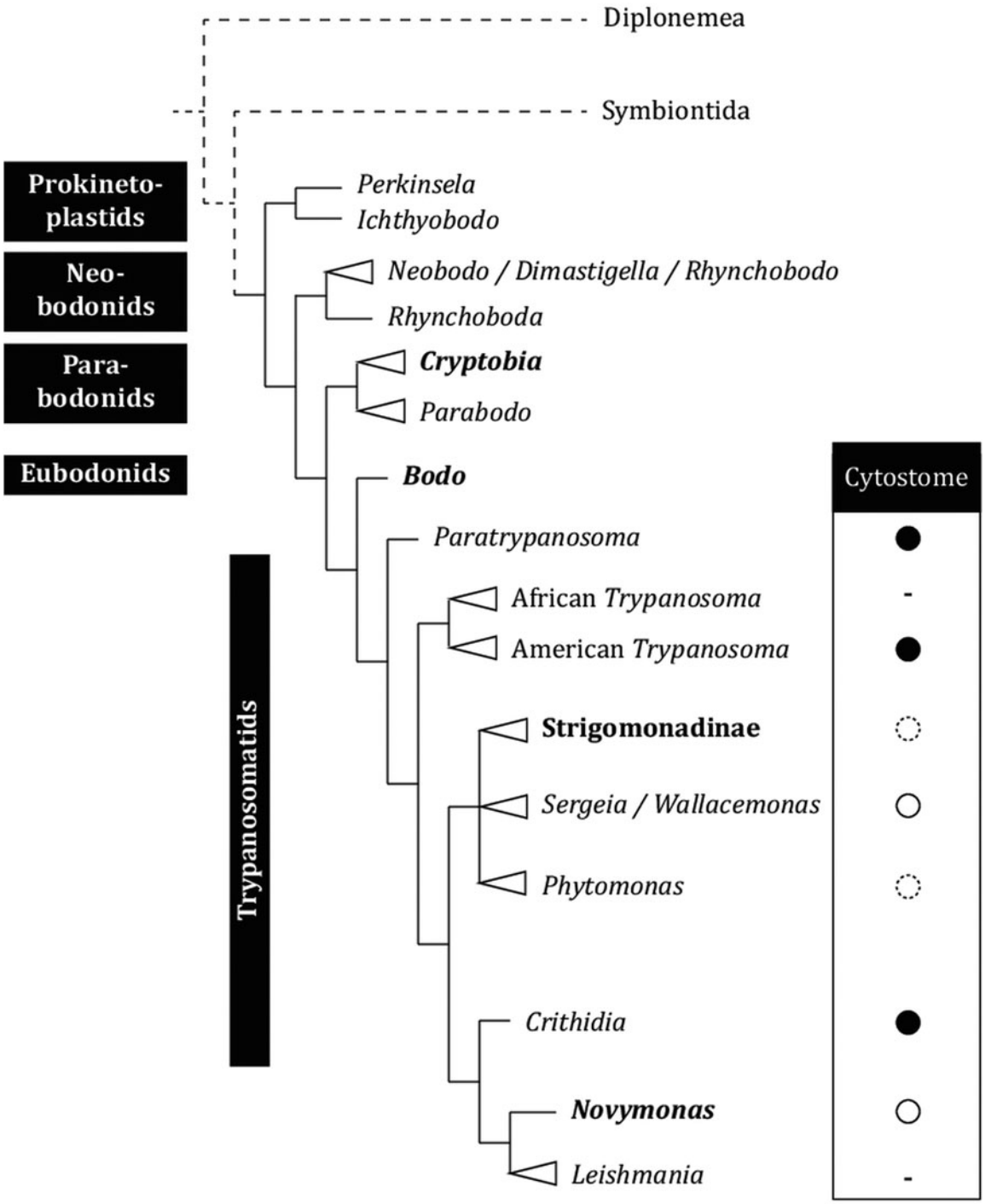

Fig. 1. Kinetoplastid phylogeny and a history of endosymbiosis. Taxa in possession of bacterial endosymbionts are highlighted in bold. Filled circles denote presence of a cytostome–cytopharynx complex in some trypanosomatid taxa; open and dashed circles denote uncertainty (as defined by an absence of data) or an unlikeliness (based on extensive, published electron microscopy studies), respectively, with regard to the presence of these structures in others; – denotes absence of a cytostome–cytopharynx from *Leishmania* and African trypanosome species.

the host cell (Motta *et al.*, 2010), whereas in recently discovered *Novymonas* less stringent regulation on the number of *β*-proteobacterial *Pandoraea* endosymbionts could reflect either symbiont farming or a snap-shot of an early transitional phase in the establishment of a novel endosymbiont–host relationship (Kostygov *et al.*, 2016, 2017). In this mini-review, we consider metabolic advantages conferred by bacterial endosymbionts to their partner trypanosomatids, how the biology of the host cell potentially influences the establishment, reductive evolution and subsequent entrainment of the endosymbiont(s), and we survey the literature with regard to endosymbioses within free-living phagotrophic kinetoplastids. Finally, we also consider the fascinating example of *Perkinsela*, a basal kinetoplastid and itself an endosymbiont of *Paramoeba* sp. (Dyková *et al.*, 2003; Tanifuji *et al.*, 2011). Here, the evolutionary path from protist to obligate endosymbiont has been accompanied by streamlining and loss of much cell biology that defines and characterizes the Kinetoplastea (Tanifuji *et al.*, 2017).

Independent origins of endosymbiosis among trypanosomatids

Species belonging to four trypanosomatid genera, spanning two monophyletic groups (Fig. 1), are characterized by the presence

https://doi.org/10.1017/S0031182018000781

of a bacterial endosymbiont. Phylogenetic analyses indicate *Novymonas esmeraldas*, the most recently characterized endosymbiont-bearing trypanosomatid isolated in Ecuador from a scentless plant bug (*Niesthrea vincentii*) (Kostygov *et al.*, 2016), is closely related to the genus *Leishmania* that encompasses more than 30 species of dixenous parasites found variously in tropical and sub-tropical countries across the New and Old World and include the aetiological agents of cutaneous, mucocutaneous and visceral human disease (Akhoundi *et al.*, 2017). *Novymonas esmeraldas* is considered to be monoxenous and non-pathogenic, despite its close relatedness with *Leishmania*. It contains a *β*-proteobacterial endosymbiont, *Candidatus* Pandoraea novymonadis (order Burkholderiales; family Burkholderiaceae) (Kostygov *et al.*, 2017). Environmental DNA reads corresponding to 18S rRNA and trypanosomatid spliced leader RNA gene sequences point to the presence of trypanosomatid taxa very closely related to *N. esmeraldas* in Central Africa (Kostygov *et al.*, 2016), raising the question of whether such taxa also contain similar *Pandoraea*-related endosymbionts.

In contrast, strigomonads form a discrete monophyletic clade most closely related to the genera *Wallacemonas* and *Sergeia* and some distance removed from the leishmanias (Teixeira *et al.*, 2011; Votýpka *et al.*, 2014). In the common ancestor of the three genera-forming Strigomonadinae – *Angomonas*, *Strigomonas* and *Kentomonas* – an endosymbiotic association with a different member of the Burkholderiales (family Alcaligenaceae) occurred. Considered to be a more ancient relationship than the endosymbiosis occurring in *N. esmeraldas*, the cell cycle of the endosymbiont in strigomonads is firmly entrained within that of its host cell. The peptidoglycan and outermost layers of the endosymbiont cell envelope are absent or heavily reduced (Motta *et al.*, 1997; de Souza and Motta, 1999), potentially facilitating easy metabolite transfer between host and endosymbiont (discussed further in the 'Interface with host cell biology'). Bacterial endosymbionts in Strigomonadinae are known as *Candidatus* Kinetoplastibacterium spp. (Alves *et al.*, 2013). The known distribution of strigomonads is also more cosmopolitan than that of *Novymonas*, as they are variously found in heteropteran and dipteran insects. Moreover, different *Angomonas* species have been isolated from Europe, the Americas, Africa and Australia, while *Kentomonas* has been isolated from Ecuador and the Philippines, and *Strigomonas* was encountered in different regions of the Americas (Maslov *et al.*, 2013; Votýpka *et al.*, 2014).

Classic hallmarks of the transition to an obligate endosymbiotic life cycle are evident in all trypanosomatid endosymbionts: a reduced GC-content (in comparison with free-living relations), reduced genome size, a paucity of mobile elements, and a reduced gene content (Table 1). Among different *Candidatus* Kinetoplastibacterium spp. from the strigomonads there are only slight variations in overall gene content and near-complete preservation of synteny (Alves *et al.*, 2013; Silva *et al.*, 2018) indicating reductive evolution of these endosymbionts had progressed nearly to completion prior to the divergence of the last common *Strigomonas*/*Angomonas*/*Kentomonas* ancestor(s) or that reductive evolution of endosymbionts followed parallel trajectories in each strigomonad lineage (Alves *et al.*, 2013). We pick up briefly in the discussion of the 'Interface with host cell biology' how the reductive evolution of trypanosomatid endosymbiont gene content commonly incorporates loss of processes associated with a free-living lifestyle and perception of environmental change.

Common metabolic gains in endosymbiont-containing trypanosomatids

A consequence of any endosymbiosis is conferment of new metabolic capability for the host cell. Taken to extremes, an endosymbiont's cell cycle can become entrained within that of its host and the advent of translocon-mediated protein targeting from host to endosymbiont classically marks the transition from endosymbiont to 'organelle' (Cavalier-Smith and Lee, 1985; Theissen and Martin, 2006; Keeling *et al.*, 2015; McCutcheon, 2016). Among eukaryotes, the most easily recognizable products of endosymbiotic relationships are mitochondria, which conferred cytochrome-dependent oxidative phosphorylation upon an archaeal host cell of ill-defined metabolic capability (Sousa *et al.*, 2016; Eme *et al.*, 2017; Zachar and Szathmáry, 2017), and chloroplasts responsible for photosynthesis; they have evolved independently twice as the consequence of a primary endosymbiotic event (Nowack and Grossman, 2012; Singer *et al.*, 2017). These organelles were pivotal in the radiation of eukaryotic diversity with chloroplasts, notably of red algal origin, also becoming widely established in many protist lineages as consequences of secondary and tertiary endosymbiosis (Keeling, 2013). On a global scale, chloroplast functions remain integral to carbon cycle dynamics (Pan *et al.*, 2011; Phillips and Lewis, 2014; Worden *et al.*, 2015). At a species level, a few taxa are also secondarily photosynthetic owing to transient retention of chloroplasts (and transcriptionally active nuclei) from their algal prey (Dorrell and Howe, 2012). This phenomenon is termed 'kleptoplastidy'; such opportunistic oxygenic photosynthesis potentially confers several advantages, including aerobic respiration within anoxic environments (Esteban *et al.*, 2009). A wide variety of other endosymbioses also exist in eukaryotic evolution that confer alternative physiological advantage(s) for the host cell as consequences of different metabolic gains, e.g. N_2 fixation and N_2 recycling (from waste host urea, ammonium products) occurring in termite gut-dwelling parabasalid and oxymonad flagellates and in some diatoms or, among anaerobic (non-photosynthetic) ciliates, CO_2 fixation by methanogenic bacterial endosymbionts that utilize H_2 produced as a metabolic end-product by the host cell (Nowack and Melkonian, 2010; Allen *et al.*, 2011; Carpenter *et al.*, 2013; Tai *et al.*, 2016).

Among the Trypanosomatidae, endosymbiosis likely confers physiological advantage within nutritionally challenging

Table 1. Genome properties of trypanosomatid endosymbionts and related taxa

Characteristic	*Ca.* Pan. Nov	fl *Pan.* spp.	*Ca.* Kin.	*Tay. equ*	*Ach. xyl*
Genome size (Mb)	1.16	4.46–6.50	0.74–0.83	1.70	7.36
GC-content (%)	44	63–65	25–33	37	66
No. of protein-coding genes	968	4181–5342	670–742	1556	6815
No. of pseudogenes	13	76–361	1–20	0	0
Reference	Kostygov *et al.* (2017)		Alves *et al.* (2013) Silva *et al.* (2018)		

Ca. Pan. nov, *Candidatus* Pandoraea novymonadis; fl *Pan.* spp., free-living *Pandoraea* species; *Ca.* Kin, *Candidatus* Kinetoplastibacterium; *Tay. equ*, *Taylorella equigenitalis* (pathogenic bacterium closely related to *Ca.* Kinetoplastibacterium; Alves *et al.*, 2013); *Ach. xyl*, *Achromobacter xylosoxidans* (free-living bacterium closely related to *Ca.* Kinetoplastibacterium; Alves *et al.*, 2013).

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environments offered by the digestive tracts of their invertebrate vectors (or hosts). However, it is neither N_2 nor CO_2 fixation or an ability to utilize or provide alternative carbon sources or electron acceptors for energy generation that differentiate endosymbiont-bearing trypanosomatids from other trypanosomatids. Instead, their endosymbionts render strigomonads and *Novymonas* autotrophic for vitamins (or cofactor precursors), amino acids, purines, and heme which are all essential nutrients in other trypanosomatids (Table 2). The curious exception is the endosymbiont from *Kentomonas sorsogonicus*, which is missing the haem biosynthetic pathway and the host cell is thus reliant upon an exogenous source of haem within its culture medium (Silva *et al.*, 2018). As highlighted in Table 2, in many instances complete biosynthetic pathways are encoded within endosymbiont genomes; in other instances, metabolite exchange between endosymbiont and host is required to complete amino acid, haem or vitamin provision. For *Angomonas deanei*, *Strigomonas culicis* and *S. oncopelti*, predictions for autotrophy arising from genome annotations are consistent with early descriptions of minimal culture media (Newton, 1957; Mundim *et al.*, 1974; De Menezes *et al.*, 1991).

Whether the enhanced autotrophies of endosymbiont-containing trypanosomatids serve to widen the range of vectors that can be colonized and/or offers these trypanosomatids a competitive edge over other microbiota that may compete for the gut niche is not known. At first glance, the relative rarity of endosymbiont-containing trypanosomatids in ecological surveys argues against either of these possibilities. However, it is moot whether susceptibility to antibiotics typically applied during isolation into the culture of trypanosomatids from ecological surveys limits the frequency with which endosymbiont-bearing taxa are found. Insect digestive tracts colonized by trypanosomatids are ill-understood environments, but although they clearly provide sufficient haem, purines, vitamins of the group B and other precursors to support parasite replication in different regions of the alimentary tract, they are also unequivocally nutritionally challenging environments. Several pieces of evidence support this assertion of a nutritional 'knife-edge': (i) with rare exception, trypanosomatid species present (in comparison with other parasites) complex and robust metabolic networks for central energy metabolism and anabolism (notably in the extent of sterol and other lipid biosynthetic pathways) (Ginger, 2006; Kraeva *et al.*, 2015; Opperdoes *et al.*, 2016); (ii) retention in some trypanosomatids of enzymes to (a) complete biosynthetic pathways for which gut microbiota can provide initial precursors – e.g. the importance of homoserine kinase coupled to the expression of threonine synthase in tsetse-dwelling forms of the African trypanosome *Trypanosoma brucei* (Ong *et al.*, 2015) or (b) catabolize carbon sources likely specific to the insect vectors of some trypanosomatids – e.g. histidine in the reduviid vector of the American trypanosome *T. cruzi* (Berriman *et al.*, 2005); (iii) the extensive reductive evolution of central metabolism that does occur in trypanosomatids when they become adapted to live in particularly nutrient rich environments – e.g. *Phytomonas* in sugar-rich plant sap (Kořený *et al.*, 2012; Porcel *et al.*, 2014) or kinetoplast loss in mechanically – rather than tsetse-transmitted African trypanosomes (Lai *et al.*, 2008). Intriguingly, the loss of respiratory complexes III and IV in *Phytomonas* (Nawathean and Maslov, 2000) may have helped facilitate an ability of *P. françai* to colonize its cyanide-rich cassava host. Comparative analysis of proteome and annotated genomes of endosymbiont-containing *A. deanei* and *S. culcis* have indicated no obvious moderation of the central metabolic networks seen in better studied *Leishmania* or *Trypanosoma* parasites (Motta *et al.*, 2013).

Table 2. Metabolic gains for endosymbiont-containing trypanosomatids

Metabolic gain	RT	*Ne*	*A/K/S*
Haem biosynthesis	–	+	+[a]
Purine provision	–	+	+
Branched chain a.a. synthesis (leu, iso, val)	–	+	+[b]
Aromatic a.a. synthesis (phe, trp, tyr)	–	+	+
Lys biosynthesis	–	+	+
De novo folic acid production	–	+	+
Thiamine, nicotinic acid, biotin provision	–	+	–
Riboflavin, pantothenic acid, vitamin B_6 provision	–	+	+[c]

RT, regular trypanosomatids; *Ne*, *Novymonas esmeraldas*; *A/K/S*, *Angomonas*/*Kentomonas*/*Strigomonas*.
[a]With the exception of the endosymbiont from the sole characterized *Kentomonas* species (*K. sorsogonicus*) where the haem biosynthetic pathway is absent from both host and its endosymbiont (Silva *et al.*, 2018).
[b]Requires use of host cell branched-chain amino acid aminotransferase.
[c]Pantothenic acid synthesis utilizes enzymes from host and endosymbiont.

Interface with host cell biology I: strigomonads the slavers; *Novymonas* the farmer

Despite some variations in cell shape, all endosymbiont-containing trypanosomatids adopt liberform morphologies where the flagellum is not attached for an extended region to the cell body following exit from the flagellar pocket (Fig. 2).

In strigomonads, their endosymbiont is positioned proximate to the nucleus and its replication and division in the cell cycle entrained (Motta *et al.*, 2010): endosymbiont duplication occurs early in the cell cycle preceding the host cell's discrete kinetoplast S-phase and segregation, which is coupled to flagellar basal body segregation (Ogbadoyi *et al.*, 2003); endosymbiont division is followed by movement of the endosymbionts such that each is positioned on opposite outer-faces of the nucleus; mitosis (with each nucleus associated with a single endosymbiont) and new flagellum elongation beyond the flagellar pocket exit point conclude the latter stages of the cell cycle prior to cytokinesis. Annotation of *Ca.* Kinetoplastibacterium genomes reveal they lack much of the machinery associated with bacterial cell division, indicating involvement from the host cell in that regard (Alves *et al.*, 2013; Motta *et al.*, 2013). The co-ordination of endosymbiont division within that of the host cell is illustrated further by the effect of the addition of aphidicolin, an inhibitor of eukaryotic replication DNA polymerases, or the eukaryotic translation inhibitor cycloheximide to *A. deanei* or *S. culicis* (Catta-Preta *et al.*, 2015). Application of either eukaryotic growth inhibitor resulted in cessation of host cell growth and division and also blocked endosymbiont division but not endosymbiont replication. Application of aphidicolin in *S. culicis* additionally caused filamentation of bacteria indicating re-entry of the endosymbiont into subsequent cell cycles and continued DNA replication but without any completion of cytokinesis (Catta-Preta *et al.*, 2015).

Candidatus Pandoraea novymonadis replicates more readily within the cytoplasm of its host cell (Fig. 3A and B). In multiplicative *N. esmeraldas* promastigotes, ~70% of the population contain between two and six endosymbionts, with 10 or more present in ~5% of cells (Kostygov *et al.*, 2016). Approximately 6% of *Novymonas* cells are aposymbiotic although the extreme difficulty in cloning such cells, the retention of intracellular bacteria in cultures since their isolation, and the significant deceleration of an aposymbiotic cell line growth as compared with wild-type highlight the importance of *Ca.* Pandoraea to host cell fitness (Kostygov *et al.*, 2017). This contrasts with strigomonads where aposymbiotic populations, albeit replicating more slowly than parental lines and with increased nutritional

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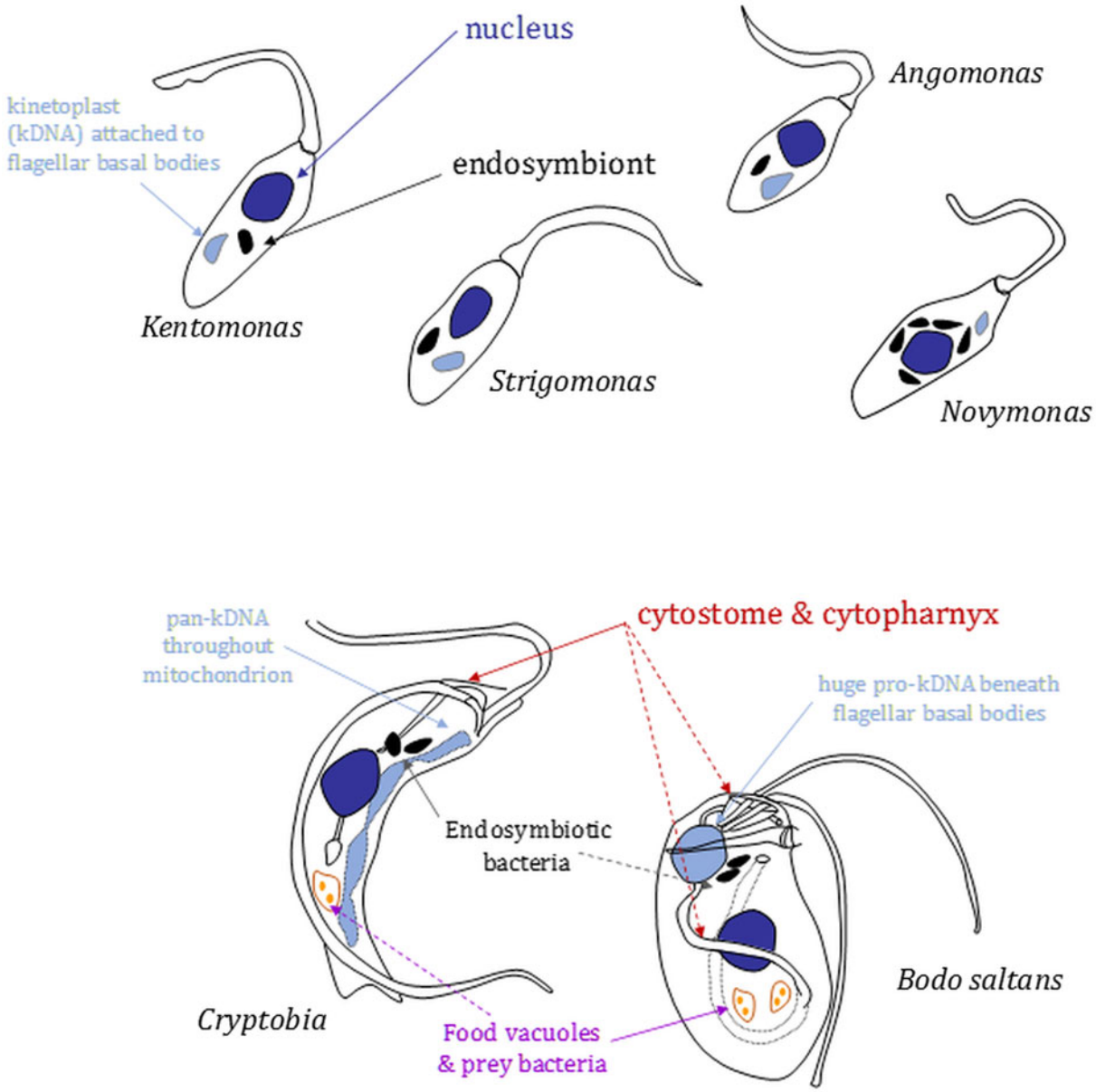

Fig. 2. Morphology and nucleus–mitochondrial genome–endosymbiont organization in endosymbiont-containing kinetoplastids. Cartoons (not to scale) are based on images shown in Kostygov *et al.* (2016), Teixeira *et al.* (2011) and Votýpka *et al.* (2014) or original drawings in Brooker (1971*a*) and Vickerman (1977). Relative positions of several organelles discussed in the main text are shown. Shading: black, bacterial endosymbionts; dark grey, nuclei; light grey, mitochondrial genomes [kinetoplasts (kDNA) or (in *Cryptobia*) pan-kDNA and (in *Bodo saltans*) pro-kDNA].

requirements, can be readily obtained by treatment of cultures with chloramphenicol (de Souza and Motta, 1999). Intriguingly, studies of aposymbiotic strigomonads reveal another possible dimension to the host-endosymbiont interface with differences evident in cell surface carbohydrate composition between symbiont-containing and symbiont-lacking *S. culicis* cultivated in equivalent media and the indication that altered surface composition negatively influences interaction of the trypanosomatid with permissive insect hosts (Dwyer and Chang 1976; Catta-Preta *et al.*, 2013; d'Avila-Levy *et al.*, 2015). Significantly, culture conditions have been shown to influence the composition of the cell surface of other trypanosomatids, demonstrating common links between nutritional status and cell surface properties (Vassella *et al.*, 2000; Morris *et al.*, 2002).

Fusion of *Novymonas* lysosomes with *Ca.* P. novymonadis provides an indication that the host 'farms' its endosymbiont, presumably taking amino acids, haem, purines and other molecules liberated in lysosomes to satisfy dietary requirements. In agreement with the 'lax' control on endosymbiont multiplication evident in *Novymonas*, *Ca.* P. novymonadis retains more genes associated with bacterial cell division than *Ca.* Kinetoplastibacterium (Kostygov *et al.*, 2017). However, other findings from *Ca.* P. novymonadis genome annotation point to a well-established host–endosymbiont relationship and provide a note of caution for any assumption of how readily *Ca.* P. novymonadis might multiply free from the host cell in different, commonly used bacterial growth media. For instance, cellular characteristics associated with perception and response to environmental change are either absent (genes for pilus and flagellum assemblies, 'wsp' chemotaxis proteins, 'pel' proteins involved in biofilm formation) or minimalized (two-component signalling). There is also a drastic reduction in the number of nutrient transporters/exporters present, including members of ABC-transporter and major facilitator superfamilies and in the ability of *Ca.* P. novymonadis to catabolize diverse carbon sources in comparison with free-living *Pandoraea* (Fig. 4).

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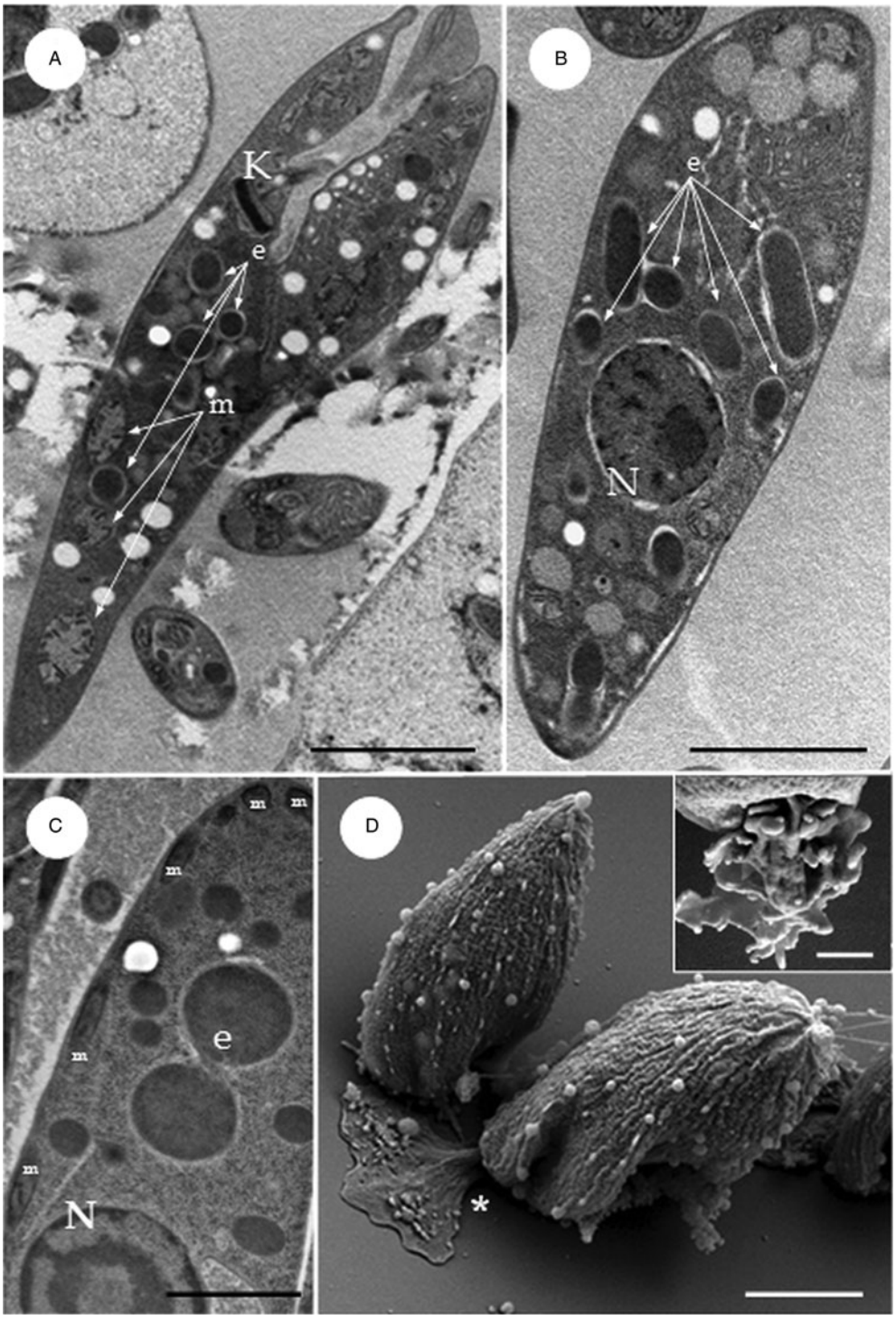

Fig. 3. Electron microscopy of the endosymbiont–host cell association and cell form in *Novymonas* and *Kentomonas*. (A and B) Longitudinal sections through *N. esmeraldas* promastigotes showing the presence of multiple endosymbiont profiles (e). Also highlighted are the kinetoplast (K), nucleus (N) and cross-sections through the mitochondrion (m). (C) Longitudinal section through a *Kentomonas sorsogonicus* choanomastigote illustrating (i) a dividing bacterial endosymbiont and (ii) mitochondrial hypertrophy and loss of typical microtubule spacing within the sub-pellicular array. (D) Sessile *N. esmeraldas* choanomastigote attached to the substrate surface *via* a modified flagellum (asterisk). Inset, the modified flagellum of a sessile choanomastigote revealing a possible open collar structure to the flagellar pocket exit point. Scale bars (A) and (B) 2 μm; (C) 1 μm; (D) 2 μm (inset, 400 nm). Images in (D) are reproduced from Kostygov *et al.* (2016) under the terms of a Creative Commons Attribution-Noncommercial-ShareAlike 3.0 Unported licence.

However, metabolic dependencies in endosymbiotic relationships go both ways. In the trypanosomatid examples, owing in large part to the close proximity of strigomonad endosymbionts to host cell mitochondria and glycosomes, strigomonads have for many years been considered to provide ATP to their intracellular partners (see Loyola-Machado *et al.*, 2017 for recent consideration of this topic). This assertion is supported by the paucity of options for efficient oxidative phosphorylation by *Ca.* Kinetoplastibacterium spp.

Genomes of both *Ca.* Kinetoplastibacterium and *Ca.* P. novymonadis contain genes for *nuo*-type NADH:ubiquinone oxidoreductases (Kostygov *et al.*, 2017), but in the former its electron transport chain is truncated to a cytochrome *bd* terminal oxidase for transfer of electrons from ubiquinone to O_2 – the type of terminal oxidase favoured by numerous bacteria, including *Escherichia coli* under low O_2 availability. In contrast to *Ca.* Kinetoplastibacterium spp., however, whilst the carbon source(s)

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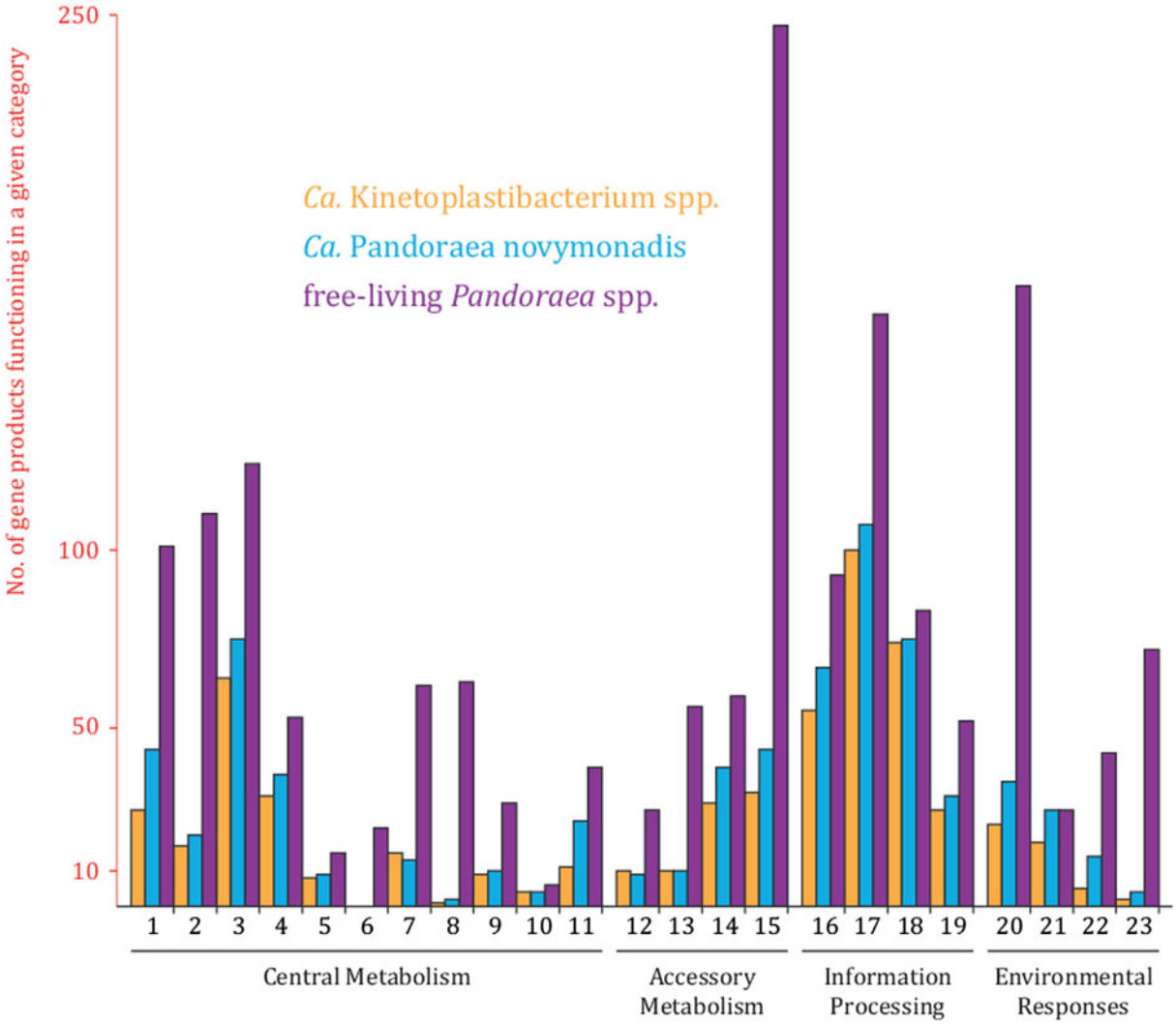

Fig. 4. *In silico* annotated proteomes illustrate the reductive evolution of *Ca.* Pandoraea novymonadis and *Ca.* Kinetoplastibacterium. Predicted protein repertoires for *Ca.* P, novymonadis, 5 *Ca.* Kinetoplastibacterium spp. and 11 free-living *Pandoraea* species (Kostygov *et al.*, 2017) were analysed according to within the KEGG Orthology (KO). 2728 KO functions were analysed. For *Ca.* Kinetoplastibacterium spp. and *Pandoraea* spp. annotation of gene products in 3 or 5 genomes, respectively, were required for inclusion in the chart shown. Known nearest free-living relatives of *Ca.* Kinetoplastibacterium are evolutionarily more distant than for *Ca.* P. novymonadis, and were not therefore included in the analysis although we note the closest *Ca.* Kinetoplastibacterium free-living relative, *A. xylosoxidans*, is more gene-rich than free-living *Pandoraea* spp. (Table 2). Individual gene products were scored once and appear in only one of the following categories. *Central metabolism*: category 1, carbohydrate usage (including lipopolysaccharide and peptidoglycan assembly); 2, amino acid catabolism; 3, amino acid biosynthesis (including glycolysis); 4, fatty acid and terpenoid metabolism; 5, inositol phosphate and glycerophospholipid metabolism; 6 butanoate and propanoate metabolism; 7, pyruvate, glyoxylate, and dicarboxylate metabolism; 8, degradation of aromatics; 9, pentose phosphate and antioxidant metabolism; 10, Krebs cycle; 11, respiration and oxidative phosphorylation. *Accessory metabolism*: 12, porphyrin metabolism; 13, miscellaneous (including carbon fixation, sulphur and methane metabolism, urease); 14, vitamin and cofactor biosynthesis; 15, transporters and ATPases. *Information processing*: 16, replication and DNA repair; 17, purine and pyrimidine metabolism (including tRNA processing and core transcription); 18, ribosome and translation; 19, chaperones. *Environmental responses*: 20, two-component signaling, transcriptional regulation, quorum sensing and phosphate metabolism; 21, cell division; 22, secondary metabolism and antibiotic defence/attack; 23, flagellum, pilus, biofilm formation.

utilized by *Novymonas* endosymbionts remains enigmatic – fructose, common in the diet of plant-feeding insects, is the most likely carbon source (Kostygov *et al.*, 2017) – the novymonad endosymbiont appears more self-sufficient for energy generation. Perhaps as a consequence of their greater autonomy with regard to their rate of cell division, and thus a greater need for intra-symbiont ATP generation, *Ca.* P. novymonadis retains a more expansive electron transport chain. Here the metabolism includes a capacity for oxidative phosphorylation from *c*-type cytochrome-dependent respiration.

Currently, the least explored facet of the interface from host to endosymbiont is the degree to which the host cell targets nuclear-encoded proteins to the symbiont. One example is known for *A. deanei* (Morales *et al.*, 2016*b*), but this is a long way short of the number of host-targeted proteins that might be required to question whether trypanosomatid endosymbionts begin to blur boundaries between endosymbiont and organelles.

Interface with host cell biology II: symbiont acquisition by closed-mouth, osmotrophic trypanosomatids – how?

In contrast to phagotrophic bodonids and other free-living kinetoplastids, trypanosomatids are obligate osmotrophs. A robust sub-pellicular mono-layer of microtubules cross-linked to one another and the over-laying plasma membrane provides a corset that defines characteristic trypanosomatid cell morphologies and prevents general endocytosis or membrane invagination across the cell surface. Membrane invagination occurs only at points where the sub-pellicular corset is absent which, in well-studied African trypanosomes and *Leishmania*, is where the flagellar pocket forms around the single flagellum emerging from the cell body. In these trypanosomatids, the flagellar pocket is the site of endo- and exocytic traffic (Field and Carrington, 2009). At the flagellum exit point, an essential collar marks the flagellar

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pocket boundary (Bonhivers *et al.*, 2008) limiting the size and rate of macromolecular traffic into the pocket lumen (Gadelha *et al.*, 2009). Given these constraints, how, following radiation of various trypanosomatid lineages, have trypanosomatid–endosymbiont associations occurred on at least two occasions?

Several possibilities can explain the conundrum of how *Novymonas* and a strigomonad ancestor acquired their respective bacterial endosymbionts. Conserved in free-living kinetoplastids and present in some trypanosomatids (Brooker, 1971*a*, 1971*b*; Brugerolle *et al.*, 1979; Attias *et al.*, 1996; Alcantara *et al.*, 2017; Skalický *et al.*, 2017) is a cytostome–cytopharynx complex, sitting in close proximity to the flagellar pocket (Figs 1 and 5). In *T. cruzi* (Porto-Carreiro *et al.*, 2000) and apparently in *Crithidia fasciculata* (Brooker, 1971*b*) the cytostome is a site of endo- and pinocytosis. In free-living kinetoplastids, the cytostome leading to the cytopharynx, in conjunction with the anterior flagellum, is used for phagotrophic feeding on bacterial prey. Early microscopy analyses indicate extensive distension of the feeding apparatus in order to ingest large prey (Brooker, 1971*a*; Burzell, 1973, 1975). Enzymatic machinery necessary for digestion of complex macromolecular structures from live prey is considered to have been lost at an early point following divergence of the last common trypanosomatid ancestor (Skalický *et al.*, 2017), coincident with the advent of obligate osmotrophy but also indicating that fortuitous uptake of a bacterium by a cytostome-bearing trypanosomatid would not necessarily be followed by its digestion.

Although clearly absent from African trypanosomes and *Leishmania* (Skalický *et al.*, 2017), a paucity of data cannot

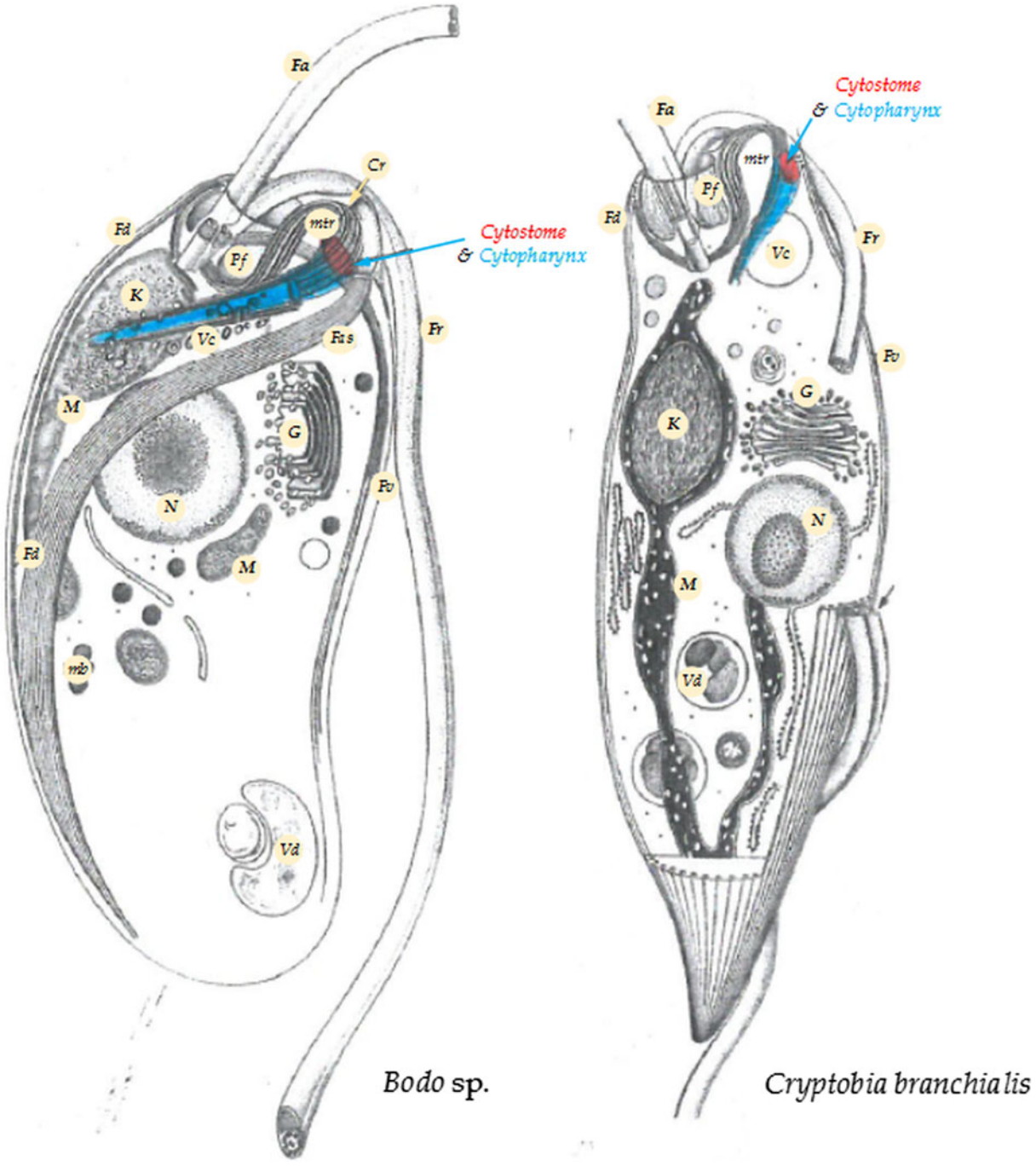

Fig. 5. Relative positions of flagella, cytostome, cytopharynx and other cellular features in free-living *Bodo* and *Cryptobia* kinetoplastids. Images were adapted from original drawings in Figs 4–6 from Brugerolle *et al.* (1979). Abbreviations (translated from the original French): *Cr*, oral ridge; *Fas*, 'microtubule fibre' associated with the 'striatal plaque'; *Fd*, 'dorsal fibre'; *Fr*, recurrent flagellum; *Fv*, 'ventral fibre'; *Fa*, anterior flagellum; *G*, Golgi; *K*, kintetoplast; *M*, mitochondrion; *mb*, microbodies; *mtr*, 'reinforced microtubules; *N*, nucleus; *Pf*, flagellar pocket; *Vc*, contractile vacuole; *Vd*, food vacuole.

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yet allow insight into how often and when the cytostome–cytopharynx was lost during trypanosomatid evolution. Whilst this organelle complex has never been seen from detailed ultrastructural analyses of extant strigomonads (Bombaça *et al.*, 2017; Loyola-Machado *et al.*, 2017) or analysis of *Phytomonas* sp. (e.g. Postell and McGhee, 1981; Milder *et al.*, 1990) and functionality of the *Crithidia* 'cytostome' has not, to our knowledge been revisited since the early 1970s, the critical questions are whether an ancestral cytostome was present and could have played a role in endosymbiont uptake by strigomonad and/or novymonad ancestors. A cytostome–cytopharynx is retained in the basal trypanosomatid *Paratrypanosoma confusum* (Skalický *et al.*, 2017); coupled to the monophyly of the trypanosomes, plus the relatively close relationship between the leishmanias and *C. fasciculata*, the pattern of organelle degeneration and thence loss was likely complex. The observation that cytostome–cytopharynx assembly in *T. cruzi* is stage-regulated (Vidal *et al.*, 2016) also leaves open the possibility of a cryptic or hidden cytostome in other extant trypanosomatids. Thus, cell entry *via* a cytostome is a plausible route for the acquisition of *Novymonas* or strigomonad endosymbionts.

To consider alternative acquisition routes, a hypertrophied mitochondrion is a diagnostic trait for the Strigomonadinae and its invasion of the spacing between sub-pellicular microtubules (Fig. 3C) is often considered to be a consequence of endosymbiosis with the ATP requirements of the endosymbiont driving mitochondrial expansion and an increased rate of energy generation by the host cell. Looser organization of the kinetoplast, relative to other trypanosomatids, is another strigomonad-specific characteristic (Teixeira *et al.*, 2011; Votýpka *et al.*, 2014), conceivably facilitates high rates of mitochondrial gene expression, and, thus, potentially an enhanced capacity for oxidative phosphorylation relative to some other trypanosomatids (careful, cross-species quantitative assessment of metabolic rate as a function of growth rate(s) under equivalent conditions will be necessary to determine if this is the case). Considered less often, however, is the possibility that mitochondrial hypertrophy and/or disruption of sub-pellicular microtubule spacing preceded endosymbiont acquisition. In this instance, a release of constraints on plasma membrane invagination would facilitate another route for endosymbiont uptake in the ancestor of the Strigomonadinae.

Looking further at the influence(s) of mitochondrial hypertrophy, rather than the endosymbiont itself might exert on host cell biology, then another strigomonad synapomorphy is the extensive reduction of paraflagellar rod (PFR) architecture. This results in a vestigial structure extended along only the proximal third of the axoneme (Gadelha *et al.*, 2006). Reductive PFR evolution was driven, at least in part, by the loss of genes encoding the major PFR2 protein. The extreme alteration of PFR form is intriguing not least because of the essentiality of this flagellar structure in other trypanosomatids (Maga *et al.*, 1999; Ginger *et al.*, 2013; Lander *et al.*, 2015). If the view that the PFR provides an important function in maintaining intraflagellar nucleotide homeostasis is correct (Pullen *et al.*, 2004; Ginger *et al.*, 2008), then a significant increase to the efficiency of mitochondrial ATP production in strigomonads could have provided a selective driver for the enigmatic reduction of PFR form seen in this trypanosomatid group.

Mitochondrial hypertrophy is not so evident in *Novymonas* with sub-pellicular microtubules having a spacing reminiscent of that found in most trypanosomatids. Acquisition of its symbiont is thus unlikely to have occurred *via* invagination of the plasma membrane. Sessile *N. esmeraldas* choanomastigotes, however, attach to surfaces *via* their flagellum and attached in this way exhibit a drastically altered flagellum structure (Fig. 3D) reminiscent of the flagellum surface attachment remodelling seen also in *P. confusum* (Skalický *et al.*, 2017). In scanning electron micrographs of detached *N. esmeraldas* choanomastigotes (Fig. 3D; inset) the altered flagellum morphology hints at a more open flagellar pocket collar through which a flagellum membrane-attached bacterium could putatively be ingested.

Endosymbioses within free-living phagotrophic kinetoplastids

There is currently sparse data with regard to endosymbionts and their role(s) in free-living phagotrophic kinetoplastids. This is not surprising given that attention to their molecular cell biology using modern approaches is only recently forthcoming (Gomaa *et al.*, 2017). However, constraints that leave the conundrum of how at least two trypanosomatids acquired their endosymbionts – arrayed sub-pellicular microtubules; a closed flagellar pocket – are not conspicuous among free-living kinetoplastids. Plus, there are likely significant insights to be made with regard to niche adaptation and exploitation; anoxic environments provide an obvious example with kinetoplastids being one of the few protist groups for which there is only limited evidence of adaptation (Priya *et al.*, 2008). The current lack of known anaerobic kinetoplastids contrasts with observations of obligately aerobic metabolism in trypanosomatid and *Bodo saltans* genomes that nonetheless showcases several anaerobic hallmarks (Michels *et al.*, 1997; Annoura *et al.*, 2005; Opperdoes *et al.*, 2016).

Surveying the literature indicates that the presence of endosymbiotic bacteria is not an obligate characteristic of free-living kinetoplastids, e.g. an absence from *Rhynchomonas metabolita* (Burzell, 1973). When present, however, endosymbionts are found in the anterior region of the cytoplasm or in close proximity to the nucleus of other kinetoplastids, albeit far from the posterior cell region that tends to be dominated by food vacuoles containing bacteria ingested *via* the cytostome–cytopharynx (Fig. 2) (Brooker 1971*a*; Burzell, 1975; Vickerman, 1977). Likely bacterial epibionts have been noted on the surface of *Cryptobia vaginalis* (Vickerman, 1977) and in others endosymbiont multiplication keeps pace with host cell division and a reduced peptidoglycan layer of the endosymbiont cell wall is in evidence, again indicative of the establishment of long-term endosymbioses.

Only distantly related to the kinetoplatids, but nonetheless of interest, another clade of euglenozoans – Symbiontida – is characterized by a dense layer of ectosymbiotic bacteria, present on their surface. This poorly studied group of flagellates, consisting of only three known species, inhabit low-oxygen sea environments. The function of the ectosymbionts is not known (Yubuki *et al.*, 2013).

Perkinsela: the enslaved kinetoplastid

The ancestor of *Perkinsela*, which is most closely related to the fish ectoparasite *Ichthyobodo*, is thought to have diverged early in kinetoplastid evolution (Fig. 1). Extant *Perkinsela* is an obligate endosymbiont of lobose amoebae genus *Paramoeba* (phylum Amoebozoa), which are pathogenic to a variety of marine animals, including farmed fish. GC-content in *Perkinsela* is not reduced in comparison with other sampled kinetoplastids (Tanifuji *et al.*, 2017) even though the *Perkinsella–Parmoeba* endosymbiosis is a long time established association (Sibbald *et al.*, 2017). In contrast, gene content of *Perkinsela* is significantly reduced in comparison with free-living *B. saltans* and parasitic trypanosomatids – 5252 protein-coding genes in *Perkinsela vs* 18 943 genes in *B. saltans*; 6381 in *Phytomonas* sp.; 9068 in *T. brucei* (although this includes expansion of its critical antigenic variant surface glycoprotein gene repertoire); and 8272 genes in *Leishmania major*. This reductive evolution reflects the secondary loss of much of the cell biology that characterizes kinetoplastid

https://doi.org/10.1017/S0031182018000781

cell form (Tanifuji *et al.*, 2017). *Ichthyobodo*, in contrast, displays the biflagellate morphology typical of non-trypanosomatid kinetoplastids (Grassé, 1952).

Lost from the genome of *Perkinsela* are all the genes required for basal body/flagellum assembly and architecture, together with an absence of genes encoding homologues of trypanosomatid cytoskeletal proteins. The absence of sub-pellicular microtubules relieves the constraints on the surface siting of endocytosis and leaves the endosymbiont able to readily ingest cytoplasm from the host (Tanifuji *et al.*, 2017). Metabolism of *Perkinsela* is also minimized: glycolysis occurs but obvious metabolic routes from pyruvate to acetyl-CoA are lacking; a truncated Krebs' cycle running from α-ketoglutarate to oxaloacetate likely uses a (host-derived) glutamate carbon source and provides electrons to fuel a mitochondrial respiratory chain truncated by the loss of complex I (NADH:ubiquinone oxidoreductase). It is likely that the benign environment offered by the *Paramoeba* host, with respect to carbon provision, facilitates the reductive evolution of intermediary metabolism. An absence of sterol metabolism potentially reflects either absence of sterol from endosymbiont membranes (similar to a few other eukaryotes) or a possibility that the host provides an easy availability of the ergosta- and stigmasta-type sterols found in other amoebozoans and trypanosomatids (Raederstorff and Rohmer, 1985; Nes *et al.*, 1990; Roberts *et al.*, 2003). A lack of sugar nucleotide biosynthesis possibly indicates a reduced requirement for protein glycosylation or limited need for investment in a protective cell surface glycocalyx.

Benefits arising from an intracellular lifestyle for *Perkinsela* are clear, although this is not to suggest that the lifestyle is lazy: the kinetoplastid makes a huge investment in RNA editing, perhaps as a consequence of the neutral evolutionary ratchet discussed by Lukeš (2011), for the expression of the six (essential) respiratory chain components encoded on the mitochondrial genome (David *et al.*, 2015) and the nuclear genome hints at the presence of a sexual cycle that is perhaps integrated within that of its host (Tanifuji *et al.*, 2017).

Apart from *Paramoeba* and *Perkinsela*, all known endosymbioses involving only eukaryotes bring the provision of photosynthesis to the host partner (David *et al.*, 2015). What *Parameoba* derives from its unusual endosymbiont is currently a mystery and a source only for speculation.

Concluding remarks

Endosymbiosis is a feature of kinetoplastid evolution. Several case examples provide tractable opportunities to understand how, at the host–endosymbiont interface, long-lasting endosymbiotic relationships become established in microbial eukaryotes and leave other questions that will likely be more challenging to address. Of the latter, until more robust culture systems for *Paramoeba* are forthcoming, it will be difficult to establish what *Perkinsela* provides for its host. Similarly, without relevant traits being revealed in continuing surveys of trypanosomatid diversity, the chronology and interplay between endosymbiont acquisition, mitochondrial hypertrophy, altered kinetoplast structure and PFR reduction cannot realistically be addressed. However, we now work in an era of easy next-generation sequencing. Thus, paralleling combined genomic, transcriptomic and proteomic studies of environmentally sourced protists such as the breviate *Lenisia limosa* (Hamann *et al.*, 2016), there is much scope to reveal what endosymbionts (and epibiotic bacteria) contribute to free-living kinetoplastid hosts. Similarly, and in contrast to many other examples of protists with endosymbionts, the tractability of trypanosomatids towards genetic manipulation (Morales *et al.*, 2016*b*) leaves huge opportunity to dissect at a molecular level the regulatory influences on endosymbiont growth and division in strigomonads and *Novymonas*. Genetic tractability also provides the means to probe the extent to which protein targeting from host-to-symbiont (and perhaps *vice versa*) also eclipses the biology of trypanosomatid–endosymbiont associations.

Financial support. This work received support from the Czech Grant Agency (16-18699S to J.L.) and the European Regional Development Fund [project CePaViP (OPVVV16_019/0000759 to J. L. and V. Y.)].

Conflict of interest. None.

Ethical standards. Not applicable.

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