

Opinion

Evolving Differentiation in African Trypanosomes

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Differentiation is a central aspect of the parasite life cycle and encompasses adaptation to both host and environment. If we accept that evolution cannot anticipate an organism's needs as it enters a new environment, how do parasite differentiation pathways arise? The transition between vertebrate and insect stage African trypanosomes is probably one of the better studied and involves a cell-cycle arrested or 'stumpy' form that activates metabolic pathways advantageous to the parasite in the insect host. However, a range of stimuli and stress conditions can trigger similar changes, leading to formation of stumpy-like cellular states. We propose that the origin and optimisation of this differentiation program represents repurposing of a generic stress response to gain considerable gain-of-fitness associated with parasite transmission.

Stress Responses and Resource Management

Adaptation to environmental change is an essential requirement for all living organisms, albeit with a wide diversity in scope and mechanism. Broadly represented examples of extreme responses include heat shock and autophagy, both significant defences against sudden adversity and significantly associated with heat shock proteins (HSPs), well known agents mediating evolutionary change. In the case of unicellular parasites, changed environments can encompass the colonisation of different tissues within a host, as well as the transmission between hosts. The strategies employed by many eukaryotes, including pathogenic forms, in response to environmental changes include sporulation, induction of the sexual cycle, and/or development of partly quiescent forms [1–4].

For example, nutrient and environmental sensing are intimately linked in yeasts and are critical checkpoints controlling differentiation and pathogenicity under adverse conditions [5,6]. *Saccharomyces cerevisiae* interacts with a large number of species, from bacterial communities to metazoans, as well as engaging with within-species signalling [7], and even bacterial-initiated prion-mediated metabolic reprogramming, which reduces ethanol production during fermentations [8]. Positioning of genes at the nuclear periphery is involved with exploiting different carbon sources, acting as a memory of past environments and, in essence, anticipation of likely new conditions [9,10]. However, the most cogent example and probably best characterised of these pathways is sporulation, which occurs under poor nutrient conditions. Both glucose and nitrogen are inhibitors but acetate, a somewhat less rich carbon source, can activate the pathway and may accumulate as resources are depleted. Sporulation functions through the activation of meiotic-specific promoters that are poorly conserved even amongst fungi. Promoter activation involves chromatin modification and is modulated via successive waves of gene activation and the master transcription regulators IME1 and Ndt80 [11]. Additional metabolic sensing systems, such as the target of rapamycin (TOR) and Seh1-associated complex systems, are more widely conserved, including into trypanosomatids [12].

Any mechanism for adaptation and/or differentiation represents a resource management strategy that, if successful, promotes survival under challenging conditions. Depending on the predictability

Highlights

As multiple examples indicate that differentiation can be triggered by stress in protozoan parasites, we propose that stress response pathways are likely well suited to providing the frameworks for differentiation.

Selecting differentiation in trypanosomes as an exemplar, we discuss the parallels between the canonical differentiation pathway and recent observations of stresses from drug treatment and gene expression changes that indicate similar changes to protein expression as differentiation.

We propose a model whereby stress responses provide an original mechanism for survival in a novel environment and which, over time, become integrated with signalling pathways to increase differentiation efficiency and, hence, fitness.

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of environmental cues, **life history theory** (see [Glossary](#)) classifies strategies as **adaptive plasticity**, **bet-hedging**, or **fixed** [13,14]. Adaptive plasticity is the capacity of a single genotype to exhibit a range of distinct phenotypes, depending upon specific environmental cues [13,14], whereas bet-hedging is associated with a continuous trade-off between increased fitness under stressful conditions (e.g., transmission rates) at the expense of decreased fitness under so-called 'typical' conditions. A fixed strategy can occur because of more extreme environmental unpredictability and favours similar behaviour or phenotype regardless of conditions [13,14]. A successful strategy must ensure that, throughout the entire life cycle, a population is capable of sensing and appropriately adapting to environmental change. This can be assisted by preadaptations at the transcriptome, proteome, and/or metabolome level, or by having cohorts of genes required for adaptation capable of rapid activation.

Parasites must undergo specific differentiation processes to complete their life cycle and face a trade-off between how early they differentiate into a transmission-competent stage and how long they persist within the host. A balanced trade-off between differentiation and transmission is ultimately constrained by both biotic (e.g., host defences restricting proliferation, host resilience to infection and immune responses, co-infection, microbiome, and general health) and abiotic factors that include drug treatment, altered temperature, pH, osmolarity, oxygen tension, and dietary compounds, among others ([Figure 1](#)). Many of these factors have profound impacts on cellular energetics but can also represent highly specific stimuli.

The notion of an evolutionary process selecting for phenotypic traits as a means to achieve differentiation through the exploitation of ancient stress response pathways extends throughout eukaryotes and is observed in pathogens such as parasitic protozoa and nematodes [3,13,15,16]. Examples of a specific response to stress and a subsequent differentiation event are plentiful for protozoan parasites, including *Plasmodium* spp., *Toxoplasma gondii*, *Trypanosoma brucei*, and *Leishmania* [16–18]. For example, *Plasmodium falciparum* increases investment in gametocyte production when exposed to antimalarials such as chloroquine, a stress-response strategy leading to changes to *in vivo* differentiation frequency [19,20]. Gametocytes are the only form transmissible to the mosquito vector [21], where they undergo further differentiation into gametes in the insect midgut. This latter process, known as gametogenesis, is likewise triggered by external stimuli, including temperature and pH changes together with the presence of the metabolite xanthurenic acid in the midgut environment. Similarly, the *T. gondii* transition from highly replicative tachyzoites to the slow-growing, cyst-forming bradyzoites is regulated by the *c-myb*-like bradyzoite differentiation factor (BDF1) [21] and triggered by a range of stressful conditions, including alkaline pH, decreased cellular ATP, and drug-induced stress [21–23]. *Leishmania* promastigotes can be promoted to differentiate with multiple stimuli, including acidic pH and surface antigen agglutination. While these examples are clear evidence for using stress as an environmental cue for differentiation, and encountering a new environment is by definition stressful until full adaptation has taken place, they do not fully explain how parasites originally acquired their ability to adapt to a specific new environment and hence maximise their fitness.

The Stress and Challenge of Moving between Hosts

Within the kinetoplastida, **monoxenous** flagellates of insects clearly pre-date **dixenous** parasites [i.e., those with both an arthropod and mammalian (or plant) host]. Hence, ancestral kinetoplastids were arthropod parasites and incorporation of a period in a vertebrate host was the major transitional event in evolving a dixenous life cycle. This requires a considerable level of adaptation and while the current level of sophisticated mechanisms of tissue tropism, immune evasion, and quiescence in modern trypanosomatid pathogens are obviously more recent, some

Glossary

Adaptive (or phenotypic) plasticity:

the capacity of a single genotype to give rise to more than one phenotypic outcome in response to environmental or other challenges.

Anthropic principle: a philosophical premise in answer to the ultimately straw man question of why the universe (and hence physical constants) and our planet are what they are and are able to sustain sentient human life. Simply, the very fact of our ability to observe the universe constrains the observable universe to one where we are able to exist and hence limits physical constants to within a range compatible with life. Not to be confused with intelligent design, to which the anthropic principle is intrinsically opposed and undermines.

Bet-hedging: an adaptive strategy in which organisms experience decreased fitness within their 'normal' conditions in favour of increased levels of fitness under stressful or challenging environments.

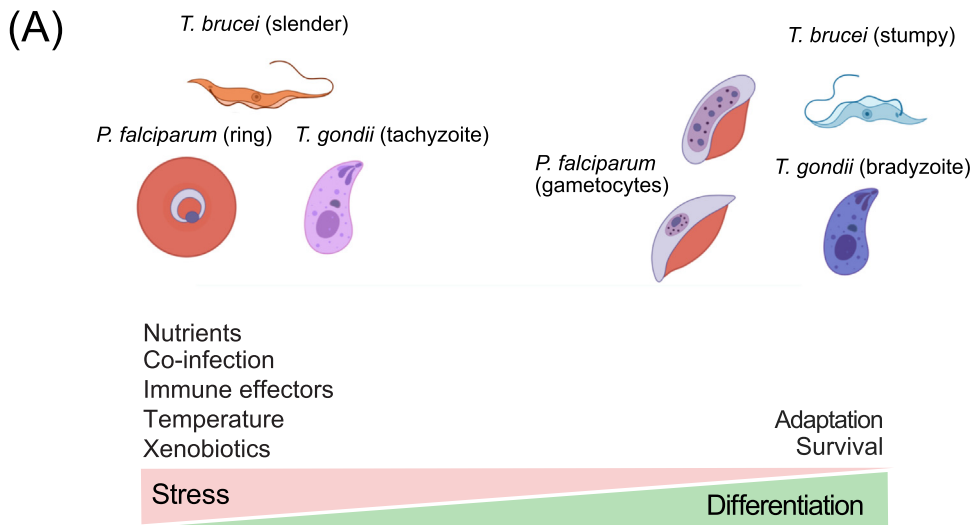
Fixed strategy: the capacity of organisms to respond and thrive in the same manner regardless of the environment or environmental challenges, albeit with some obvious limitations.

Life history theory: a branch of evolutionary theory that aims to explain how different aspects of biology, including behaviour, reproductive schedules, development, life span, and other high-order phenotypes, are shaped by natural selection. Similarly, life history theory seeks to provide a conceptual and theoretical framework to define these different strategies that organisms can exploit in response to changes in their environment.

Monomorphic and pleomorphic: literally, having only one or several forms. In the context of the present discussion, the term initially referred to the ability of certain strains to exhibit morphological variability, which was associated with a greater transmissibility. Originally recognised by David Bruce, this is now considered as connected with the ability of pleomorphic parasites to differentiate to stumpy forms significantly more efficiently than monomorphic forms.

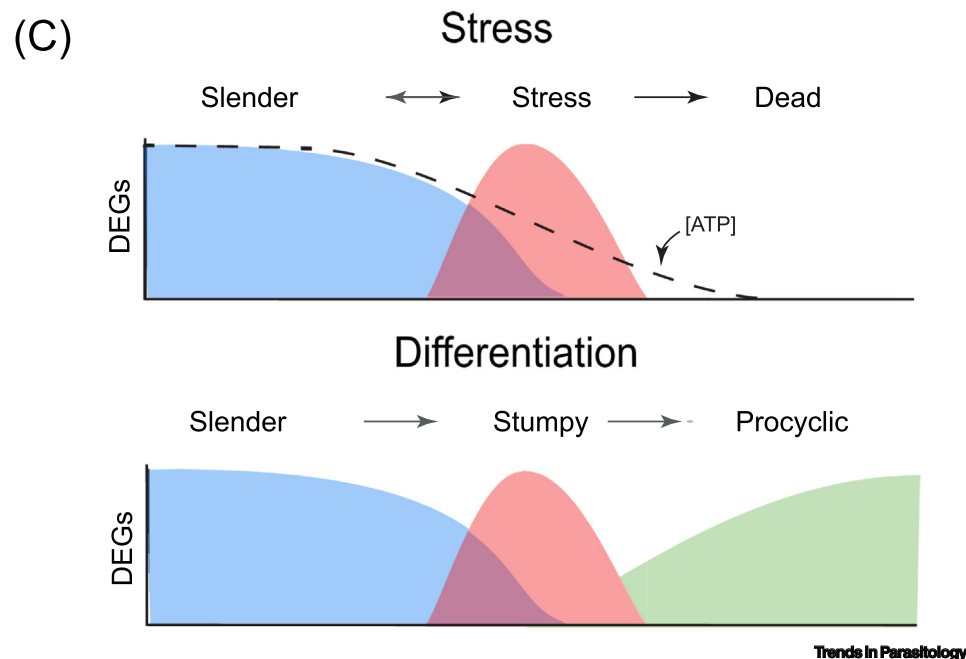
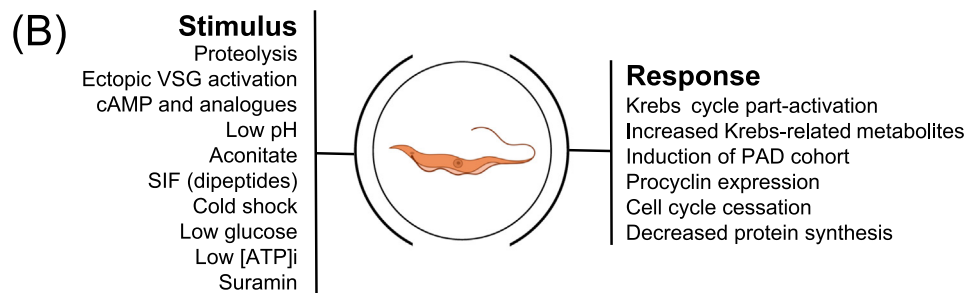
Monoxenous and dixenous: having one or two hosts within a life cycle. Traditionally a host is where the sexual cycle resides and the vector is restricted to growth of vegetative forms.

Consistent with this is that the insect is



the true host, as this is the place where meiosis is reported to occur and not the mammalian vector. Here we have used host for both insect and mammalian infections.

Stumpy: nondividing trypomastigote form cells which are 'partially' preadapted to life in the insect vector. Morphologically, stumpy cells are characterised by being shorter and rounded compared with the long, slender trypomastigotes residing in the circulation and other spaces of vertebrate hosts. Phenotypically, stumpy forms are claimed to be significantly more infective towards tsetse flies than are the slender forms, although this has been challenged.



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level of accommodation towards the new environment was essential, as was the return to the insect vector. Here, we argue that stress responses, with changes in metabolic status arose first and are fundamental for parasites (and other organisms) to thrive in a new or unexpected environment. The major triggers here are likely low cellular ATP, but that this is a generic signal and can be caused by a plethora of conditions. As these responses were, in part, providing a shift to a metabolic program that was also enabling for survival and successful proliferation within the insect vector, such stress responses were likely sufficient to gain a hold on reinfection of arthropod hosts. The transmission into a mammalian host was perhaps originally stochastic, with the return trip to the insect contingent on a sufficiently attuned stress response, which was subsequently optimised through a multifactorial selection process and connected with a signalling component. While essentially an example of the **anthropic principle**, in that trypanosomes had to have evolved successfully to be observable, stress responses are closely aligned with the need for transmission, hence facilitating life cycle completion.

Preadaptation, Differentiation, and Co-opting Stress Responses

T. brucei must successfully navigate significant changes in the environment and has exploited a rather unusual strategy as an obligate extracellular parasite, completely dependent on sophisticated immune evasion mechanisms [15,24]. Upon infection of a vertebrate host, *T. brucei* maintains a persistent infection through colonisation of multiple host tissues, including the blood, lymphatic system, skin, adipose tissue, and eventually the central nervous system [25,26]. It remains unclear how these distinct populations are coordinated or if tissue dissemination is essentially stochastic and no tropic signal has been identified [25,26]. Further, for trypanosomes residing in different host tissues, evidence for differential gene expression indicates that trypanosomes can adjust their (post)transcriptional machinery in response to specific environmental cues, for example, increased reliance on fatty acid metabolism in adipose tissue forms [26–28]. These changes are not only observed in tissue-resident trypanosomes, but also in bloodstream parasites undergoing differentiation to the **stumpy** form, triggered by a recently identified mechanism, long referred to as stumpy induction factor (SIF) [24,29–32]. This transition is also accompanied by differential gene and protein expression and is substantially better understood than the transfer from insect to mammal. Hence, we will focus our discussion on this process.

Our current understanding of the differentiation of bloodstream stage parasites into procyclic insect forms suggests a coordinated shift in gene expression, with the quiescent, nonreplicative stumpy form partially activating metabolic pathways of considerable utility in the insect [24,31,32]. These pathways include a metabolic shift from a reliance on glucose, abundant in the mammalian

Figure 1. Stress, with Benefits: Transitioning to Differentiation. (A) Both biotic (nutrient availability, temperature, immune responses, etc.) and xenobiotic factors (mainly drugs and bioactive dietary compounds) are sensed by replicating cells (left), triggering stress responses, but which potentially enable parasites to adjust to new conditions. Responses can be metabolic (*Trypanosoma brucei*), induce encystment (*Toxoplasma gondii*), or activate sexual pathways (*Plasmodium falciparum*). These derivative ‘stressed’ forms may acquire phenotypes that drive higher fitness under challenge (e.g., intermediate stumpy or quiescent bradyzoites) allowing more efficient completion of the life cycle. (B) Multiple distinct stimuli lead to many different responses. The need to integrate signals in a rational manner represents a considerable challenge in terms for understanding and unravelling those evolutionary mechanisms that give rise to differentiation pathways. (C) Differential gene expression (DEG) is associated with both stress and differentiation. The left scheme illustrates transition into a stressed state, ultimately leading to death due to decreased ATP. Coloured areas indicate specific DEG cohorts associated with individual stages. DEG was recently described for suramin-mediated killing; death occurs despite induction of metabolic pathways that potentially generate ATP and are of utility in the insect host (red). The right scheme shows a successful differentiation event; instead of death, parasites are able to survive and transition to new forms within the insect vector (red). Importantly, many genes in the red DEG cohorts are common and deletion/knockdown of these genes also leads to an inability of the stumpy form to continue to thrive in the mammalian host and hence committing to the next host. Abbreviations: PAD, protein-associated with differentiation; SIF, stumpy induction factor; VSG, variant surface glycoprotein.

bloodstream, to additional carbon sources more attuned to the composition of the midgut contents of the tsetse fly. The tsetse fly rapidly metabolises glucose taken up in a blood meal, leaving amino acids, in particular proline, as the primary carbon source for procyclic trypanosomes [33]. Other changes include an upregulation of key enzymes involved in cellular metabolism and mitochondrial activity, including Krebs cycle enzymes, NADH dehydrogenases, and ATP synthase subunits, extensively reviewed elsewhere [34–37]. The activation of this differentiation program can then be regarded as a process equipping the parasite population to survive and thrive under less lavish energetic conditions, thus enabling the passage between mammalian and insect hosts.

The transcriptional and metabolic shifts observed in the slender-to-stumpy differentiation involve the gene Tb927.8.1530 encoding for TbGPR89 [29]. TbGPR89 is an oligopeptide transporter related to the near universally conserved G protein-coupled receptor family and is expressed in replicating bloodstream forms, but is rapidly downregulated upon stumpy differentiation [29]. Secreted oligopeptidases generate a pool of very short peptides that comprise SIF and establish a paracrine signal to other cells within the trypanosome population, thereby facilitating a mechanism to control cell density [29]. The ability of bloodstream stage parasites to cleave proteins into very short peptides has been previously described for immunoglobulin degradation, although it is unknown if these two processes are connected [38]. TbGPR89 signalling depends upon RNA-binding protein 7 (RBP7), a master regulator of differential gene expression in the slender-to-stumpy transition [31].

A cohort of genes are expressed preferentially in the stumpy forms and include a ‘protein-associated with differentiation’ cohort (PAD) (Figure 1). Of these, PAD-1, a carboxylate transporter, is a convenient marker for stumpy stages [39]. Determinants controlling PAD-1 expression reside within the 3′ untranslated region of the PAD-1 mRNA, a canonical mechanism for the control of gene expression in trypanosomes [24]. Over 40 genes have been associated with these signalling pathways and recent work suggests a long noncoding RNA (lncRNA) encoded upstream of the RNA-binding protein RBP7 is important in regulating the pathway [40]. Overexpression of the lncRNA leads to premature differentiation. Furthermore, there is a kinase/phosphatase switch mechanism implicated in channelling signals via translocation of components between the flagellum and, in particular, pocket-associated endoplasmic reticulum, the glycosome, and the cytosol [41–43].

TbGPR89 acts upstream of the SIF response pathway and provides a functional link between environmental sensing, regulation of gene expression, and differentiation [20,22,24]. This is fully consistent with the hypothesis whereby trypanosomes collectively regulate their population via mechanisms reminiscent of bacterial quorum sensing and, in particular, are sensitive to population density. However, **monomorphic** parasites, considered differentiation incompetent, fail to recapitulate the stumpy phenotype observed in **pleomorphic** parasites upon inducible expression of TbGPR89 [20], indicating that in addition to TbGPR89, other factors are also necessary to fully recapitulate the reprogramming underlying differentiation. A developmental transition at high parasitaemia implies a trade-off between replication and transmission. One strategy to overcome environmental challenge is to adopt a response that both controls population density where needed and develops a state facilitating transmission into a more favourable environment. In the case of *T. brucei*, this would be another host, thereby gaining access to increased dissemination potential and resources. Given that this parallels precisely what the parasite is able to achieve, it seems plausible that *T. brucei* evolved mechanisms that merged stress responses with pathways controlling differentiation, involving quorum sensing-dependent and -independent mechanisms. In the bloodstream, trypanosomes are exposed to different types of selective pressures and stressors that they must overcome to ensure transmission. An overcrowded parasite population

would severely limit resources available to sustain an indefinite and uncontrolled population growth, triggering a global stress response. However, it is also likely that other factors might also be at play, including changes to host-derived metabolites and signalling molecules that could also trigger stress responses, for example, in tissues other than the bloodstream. In this context, it would be interesting to test whether proposed quorum sensing-mediated stumpy formation occurs in a tissue-specific manner (e.g., exclusively in the bloodstream), or if it is broadly observed in other host microenvironments. Around 20% of skin-dwelling trypanosomes are stumpy forms (based on the expression of the canonical stumpy marker PAD1) [27], where parasite densities are insufficient to trigger quorum-sensing mechanisms. Changes in local microenvironmental conditions when parasites colonise the skin and elicit events leading to differentiation are probably tissue-specific due to the relatively low parasitaemia in skin compared with the bloodstream.

All Roads Lead to Rome?

Early studies of trypanosome differentiation identified multiple signals that could stimulate these pathways, including cyclic AMP analogues, cold shock, *cis*-aconitate addition to media, and external protease treatment [30,44,45]. Some of these stimuli also act as sensitisers for other cues; for example, cold shock sensitised cells towards *cis*-aconitate/citrate, lowering concentrations required to trigger differentiation. These observations suggest the possibility of multiple signalling pathways that synergise, and possibly integrate, combinatorial information as well as generic environmental cues that trigger differentiation [45,46]. The continued status of some of the signals considered in the earlier literature is unclear; for example, the role of cAMP analogues is perhaps confused by demonstration that metabolic products are the important triggers and not cAMP or close analogues themselves [47].

Regardless, the capacity to sense nutrient availability sits at the core of the trypanosome differentiation process. For example, both cAMP and the TOR pathway work as nutrient status sensors and lead to modulation of AMP-activated protein kinase (AMPK), which has emerged as a master regulator and signaller for metabolic status, especially in coordinating cytoplasmic and mitochondrial activity. This is significant, as recent work uncovered that TOR signalling pathways, highly conserved in most eukaryotes, also operate as metabolic sensors in trypanosomes, controlling nutritional starvation and environmental stress responses [48–51]. The inhibition of AMPK blocks trypanosome differentiation in a small animal model [52], while low glucose levels can also prompt differentiation, further supporting a role for this pathway in nutritional sensing and differentiation. These environmental nutrient-sensing pathways are also crucial in the transition between hosts, characterised by a rapid drop in both glucose concentration and temperature. While trypanosomes are exposed to ~5 mM glucose in the bloodstream, this is rapidly depleted inside the insect vector upon a blood meal [53]. This severe constraint in nutrient availability can only be overcome by forms that do not rely solely on glycolysis for ATP production, including preadapted stumpy cells [36,53–55]. Similarly, culturing parasites in 5 μ M glucose, combined with cold shock, also leads to efficient differentiation, suggesting that both of these signals (nutrients and temperature) could be anticipated as being associated with the transmission event *in vivo*.

However, differentiation into stumpy forms is not exclusively regulated via cell density-dependent mechanisms [56] and two recent reports describe such events. Firstly, switching of the variant surface glycoprotein (VSG) during antigenic variation triggers a cell cycle-arrested phenotype in pleomorphic trypanosomes [56]. More specifically, during VSG replacement, activating a second VSG expression site (ES) leads to transcriptional attenuation of the previously active ES [56]. While parasites successfully acquire a new VSG coat, depending upon the level of ES attenuation, a subpopulation display growth arrest, concomitant with synchronous differentiation into stumpy (or stumpy-like) forms [56]. Moreover, these parasites could establish and sustain infection in

the tsetse fly. In a second report, analysis of trypanosome responses to suramin *in vitro* indicates overall impairment in cellular metabolism with decreased levels of cellular ATP and induction of proteins associated with stumpy differentiation, including PAD1, PAD2, PIP39, and NRKA/B [57]. Many of the proteins upregulated in suramin-treated cells are involved in mitochondrial activity and the Krebs cycle, indicating activation of metabolic pathways similar to the stumpy phenotype, albeit in this case, likely arising from an ultimately fruitless attempt to generate ATP. Omics analysis, by modelling differentiation through RBP6 overexpression, also supports this interpretation [58]. Whether suramin treatment can influence the ability to infect tsetse flies remains to be examined.

Concluding Remarks

Proliferative slender trypanosomes are, under the right circumstances, fully capable of infecting tsetse flies and triggering a canonical PAD1-mediated signalling pathway [30], supporting a view in which stumpy forms may have arisen as a consequence of stressful conditions to maximise chances of survival and transmission. Similarly, we are just beginning to understand the complexity and diversity of responses that parasites adopt in unfavourable conditions and how different environmental cues and tissue niches shape parasite populations within hosts. Much remains unclear, including how signals integrate, if other trypanosome differentiation events are prompted by stress, the generalisability of this paradigm to other organisms, and what insights we can gain from consideration of these models (see Outstanding Questions). We envision that further studies in these areas will be vital for the long-term impact of intervention strategies in infection control and eradication efforts, as well as understanding the evolution of parasite adaptive pathways.

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Outstanding Questions

How do external signals integrate in parasite differentiation?

What distinguishes a generalised stress response from targeted differentiation?

Can all trypanosome differentiation events be viewed in a similar manner and, specifically, movement from an insect to a mammalian host?

Can these pathways be manipulated for therapeutic gain?

How do stress responses relate to the origins of signalling pathways in eukaryotic cells?

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