Alexandra Isabel Vidigal Teixeira

Licenciada em Bioquímica

Understanding the role of glycerol in triggering parasite differentiation

Dissertação para obtenção do Grau de Mestre em

Bioquímica para a Saúde

Orientador: Fabien Marc Guegan, PhD

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Júri:

Presidente: Prof. Doutora Maria Teresa Nunes Mangas Catarino

Arguente: Prof. Doutora Helena Maria de Sousa Castro

Vogal: Prof. Doutor Fabien Marc Guegan

Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa

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Abstract

Trypanosoma brucei (*T. brucei*) is an extracellular parasite and the causative agent of African trypanosomiasis. The protozoan exhibits a complex life cycle taking place in an insect vector and a mammalian host and requires morphologic and metabolic adaptations in order to survive in distinct environments. *T. brucei* can occupy the bloodstream of its mammalian host as two forms: proliferative slender forms and cell-cycle arrested stumpy forms. Differentiation of slender forms to stumpy forms occurs in response to activation of a density-sensing pathway elicited by an elusive Stumpy Induction Factor (SIF) produced by the parasites.

Given that glycerol is one of the end-products of glycolysis and is naturally excreted by the parasites, in this thesis we tested whether glycerol is the SIF. We confirmed that glycerol increasingly accumulates in culture medium as parasites proliferate, correlating with greater differentiation to stumpy forms *in vitro*. Furthermore, when supplemented to the culture medium, glycerol triggers cell-cycle arrest in G0/G1 phase, induces expression of marker genes from stumpy forms, and generates stumpy forms competent to progress to the next stage of the life-cycle, the insect form.

To further elucidate the role of glycerol in promoting this developmental transition, we created two mutant parasite lines that were impaired for either glycerol production or glycerol transport across the membranes. The former was accomplished by depletion of glycerol kinase (GK) and the latter involved deletion of all aquaglyceroporin (AQP) genes. Both mutant parasite lines were incapable of accumulating glycerol extracellularly, however they did not exhibit defects in differentiation to stumpy forms. Interestingly, inhibition of glycerol production through depletion of GK induced premature differentiation to stumpy forms and dramatically reduced the parasites fitness for mouse infection. We concluded that glycerol induces differentiation to stumpy forms *in vitro* probably through a mechanism that involves accumulation of glycerol-3-phosphate inside the glycosome.

Keywords: *Trypanosoma brucei*, Differentiation into stumpy forms, Stumpy Induction Factor, Glycerol, Glycolysis

Resumo

O *Trypanosoma brucei* é um parasita extracelular e o agente etiológico da Tripanossomíase Africana. O protozoário apresenta um ciclo de vida complexo, sendo transmitido pela mosca tsé-tsé a um hospedeiro mamífero. Na corrente sanguínea pode assumir duas formas: *slender* e *stumpy*. A primeira forma é replicativa, a última é quiescente e pré-adaptada para sobreviver no vetor artrópode. A diferenciação das formas *slender* a formas *stumpy* envolve uma cascata de sinalização dependente da densidade celular, que é ativada por uma molécula produzida e secretada pelos parasitas que permanece por identificar, designada por *Stumpy Induction Factor* (SIF).

Investigámos o potencial do glicerol enquanto SIF, uma vez que este metabolito constitui um dos produtos finais da glicólise e é excretado pelos parasitas. Confirmámos que o glicerol acumula no meio de cultura à medida que a densidade dos parasitas aumenta, existindo uma correlação direta com a diferenciação a formas *stumpy*. Adicionalmente, quando este composto foi suplementado ao meio de cultura, promoveu a paragem do ciclo celular na fase G0/G1, induziu a expressão de genes característicos de parasitas *stumpy*, e ainda produziu formas pré-adaptadas para progredirem para o estadio na mosca tsé-tsé.

Para clarificar o mecanismo pelo qual o glicerol promove esta transição, criámos duas linhas celulares nas quais inibimos a produção deste metabolito ou o seu transporte através da membrana plasmática. Os parasitas de ambas as linhas mutantes não excretaram glicerol de forma significativa, no entanto a sua capacidade de diferenciação a formas *stumpy* não foi comprometida. Curiosamente, a depleção de glicerol dentro dos parasitas resultou numa maior eficiência da transformação a formas *stumpy* e afetou negativamente a capacidade destes mutantes manterem uma infeção *in vivo*. Em suma, o glicerol promove a diferenciação dos parasitas de slender a stumpy *in vitro*, provavelmente através de um mecanismo que envolve acumulação de glicerol-3-fosfato dentro do glicossoma.

Termos-chave: *Trypanosoma brucei*, Diferenciação a formas *stumpy*, Stumpy Induction Factor, Glicerol, Glicólise

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List of abbreviations

8-pCPT-cAMP – 8-(4-chlorophenylthio)-adenosine 3',5'-cyclic monophosphate

ADP – Adenosine Diphosphate

AMP – Adenosine Monophosphate

AQP – Aquaglyceroporins

ATP – Adenosine Triphosphate

bp – base pair

BSF – Bloodstream Form

cAMP – Adenosine 3',5'-cyclic Monophosphate

CDS – Coding Sequence

CM – Conditioned Medium

CNS – Central Nervous System

DHAP - Dihydroxyacetone Phosphate

DNA - Deoxyribonucleic acid

DTM – Differentiation Trypanosome medium

ES – Expression site

ESAG – Expression Site Associated Gene

G0 – Gap 0 Phase

G1 – Gap 1 Phase

gDNA – genomic DNA

GFP – Green Fluorescent Protein

GK – Glycerol Kinase

Gly-3-P – Glycerol-3-Phosphate

Glyc – Glycerol

HAT – Human African Trypanosomiasis

HMI-11 - Hirumi's modified Iscove's medium 11

HR – Homologous Region

HXK - Hexokinase

HYG – Hygromycin Phosphotransferase gene

iMM – Instituto de Medicina Molecular

KO - Knockout

LS – Long Slender

NADH - Nicotinamide adenine dinucleotide

NEO – Neomycin Phosphotransferase gene

PAC – Puromycin N-Acetyl-Tranferase gene

PAD – Proteins Associated with Differentiation

PCF – Procyclic Form

PFK - Phosphofructokinase

PGK – Phosphoglycerate kinase

PHLEO – Phleomycin Hydrolase gene

PI – Propidium Iodide

qPCR – quantitative Polymerase Chain Reaction

RNA - Ribonucleic acid

RNAi – RNA interference

rpm – revolutions per minute

RT – Room Temperature

SHAM - Salicylhydroxamic Acid

SIF – Stumpy Induction Factor

SS – Short Stumpy

TAO – Trypanosome Alternative Oxidase

TbTOR4 – *Trypanosoma brucei* Target of rapamycin 4

TCA – Tricarboxylic Acid

Tet - Tetracycline

THT – Trypanosome Hexose Transporter

UTR – Untranslated Region

VSG – Variant Surface Glycoprotein

WT – Wild-type

1. Introduction

1.1. Human African Trypanosomiasis (HAT)

Protozoa parasites *Trypanosoma brucei ssp.* (*T. brucei*) are the causative agents of Human African Trypanosomiasis (HAT) and nagana (or Animal African Trypanosomiasis) in humans and livestock, respectively. Infection is transmitted between mammalian hosts by a bite of a tsetse fly[1] (from the genus *Glossina*), being consequently restricted to sub-Saharan Africa. HAT, also known as sleeping sickness, is fatal in most cases if left untreated, and together with nagana leads to an enormous socio-economic burden, since affected rural regions depend heavily on cattle for agriculture.

The clinical features and rate of progression of HAT are dependent on which of the two *Trypanosoma brucei* subspecies is causing infection: *Trypanosoma brucei gambiense* (*T. b. gambiense*) or *Trypanosoma brucei rhodesiense (T. b. rhodesiense).* The former constitutes the most common etiologic agent of sleeping sickness and is prevalent in Central and West Africa[2,3]. The latter is found in East and Southern Africa and despite only accounting for less than 3% of the cases of HAT[4], is more virulent and provokes an acute and severe form of the condition, while infection by *Trypanosoma brucei gambiense* is chronic and exhibits a long period of latency[5]. In Uganda both subspecies overlap geographically, meaning that individuals can become co-infected[6], making diagnosis and treatment more difficult, since both subspecies are indistinguishable by microscopy and would require different trypanocidal treatments. On the other hand, *Trypanosoma brucei brucei (T. b. brucei*) is not infective to humans, usually causing illness in cattle and wild animals.

HAT can be categorized into two stages according to clinical features. Symptoms and signs of the early-stage – termed hemolymphatic stage – may include headache, intermittent fever, fatigue and pruritus. As disease progresses patients may also experience lymphadenopathy, splenomegaly, hepatomegaly, cardiac and ophthalmologic defects[7]. Additionally, weight loss is commonly observed and was recently linked to invasion of the adipose tissue by parasites[8]. The late-stage – designated meningo-encephalitic stage – involves occupation of the central nervous system (CNS) by the parasites and its leading symptom is disturbance of the sleeping pattern, also including mental perturbations and motor impairment. Investment in new treatments for HAT is needed, since the currently approved drugs for both early-stage and late-stage exhibit toxic effects, cannot be administrated orally and are sometimes ineffective[9].

1.2. *Trypanosoma brucei* life cycle

Trypanosoma brucei has a complex life cycle that comprises two distinct stages: one takes place in an insect vector (tsetse fly) and another occurs in a mammalian host (**Figure 1.1**). For that reason, they must adapt to different environments and available nutrients by changing both their morphology and metabolism[10]. Infection is transmitted between mammalian hosts upon a bite of a tsetse fly[1] carrying metacyclic forms in the salivary glands. Afterwards, parasites reach the bloodstream where they proliferate as long slender (LS) forms and occupy the interstitial spaces of several tissues (such as the adipose tissue[8]), being accountable for parasitemia and the clinical manifestations displayed in affected individuals. As the number of circulating parasites increases in the blood, slender forms begin the process of differentiating to non-replicative short stumpy (SS) forms through a density-dependent mechanism[11]. The latter are pre-adapted for transmission to the tsetse fly when ingested during a blood meal and are capable of promptly differentiating into replicative procyclic forms (PCFs) in the midgut of the insect vector[12]. The life cycle is completed when procyclic forms migrate to the salivary glands to generate metacyclic forms that can later infect new animal or human hosts. In this thesis we focused on a particular step of the life cycle of the parasite – slender to stumpy differentiation.

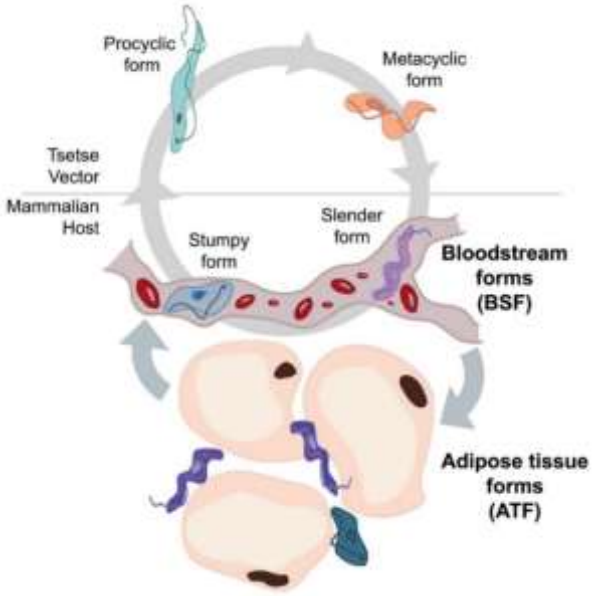

Figure 1.1 - Life cycle of *Trypanosoma brucei.* The complex life cycle of the parasite comprises two stages: one takes place in the tsetse fly, and another occurs in a mammalian host upon a blood meal of the insect vector. *T. brucei* can occupy the bloodstream of mammals as either a slender form or as a stumpy form. It was also described that parasites can functionally adapt to invade the adipose tissue of the host. Adapted from 8.

1.2.1. Differentiation of slender forms into stumpy forms *in vivo*

In the bloodstream, *T. brucei* are pleomorphic, as they can assume two distinct morphologic forms that together influence the dynamics of infection in a mammalian host (**Figure 1.2**) – slender forms and stumpy forms. African trypanosomiasis is characterized by cyclic waves of the number of circulating parasites, that are limited by two factors: the host immune system, which can clear most of the extracellular parasites, while the remaining persist through switching of their Variant Surface Glycoprotein (VSG) coat[13], thus escaping recognition; additionally,

proliferative slender forms differentiate to cell-cycle arrested stumpy forms when reaching a parasite density threshold in the blood. Parasitemia ascends again when replicative forms expressing an antigenically distinct VSG coat arise and are positively selected within the parasite population. Differentiation of slender cells to stumpy cells is mediated by a mechanism similar to *quorum sensing* in bacteria,[14,15] through which parasite density is sensed by the release and accumulation of an endogenous molecule, termed Stumpy Induction Factor (SIF)[11,12]. When a substantial amount of SIF accumulates in the bloodstream, parasites experience cell-cycle arrest and transformation to transmissible stumpy forms. Stumpy forms are essential for dissemination of sleeping sickness, since only these cells have experienced the molecular adaptations required for establishing infection in the tsetse fly and progressing to the next stage of the life cycle of *T. brucei*. Additionally, this developmental transition also extends host survival by limiting the number of proliferating parasites[13].

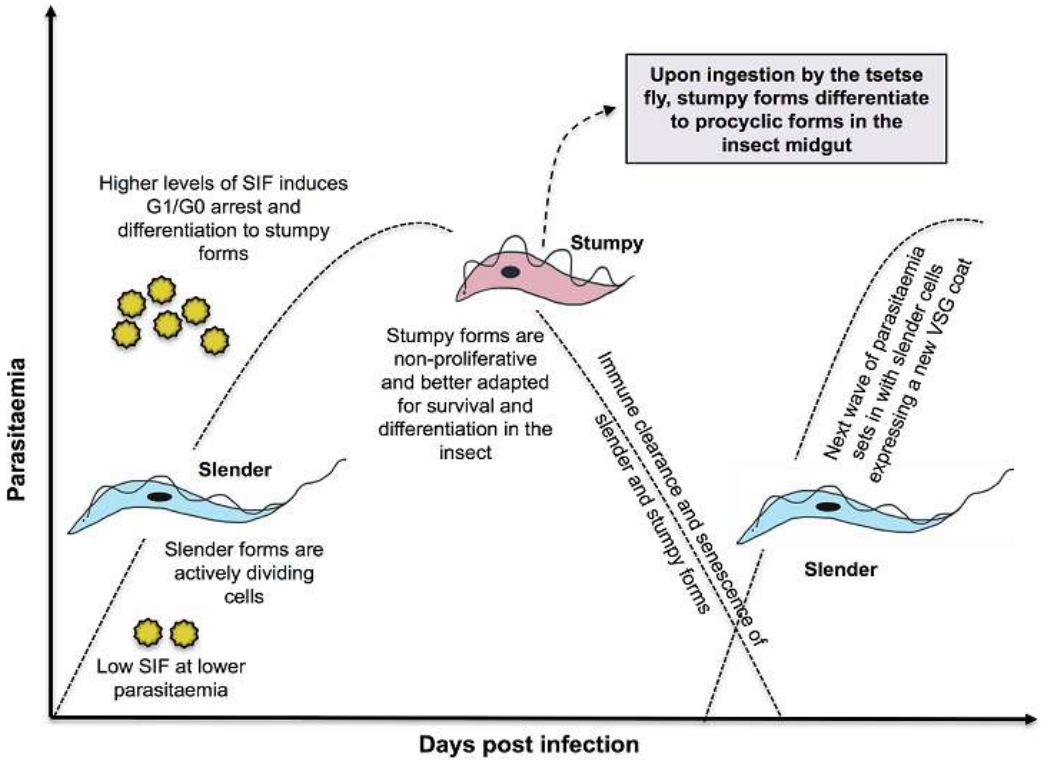

Figure 1.2 – Schematic representation of the dynamics of *T. brucei* infection in a mammalian host. Parasitemia ascends as a result of proliferation of slender forms, which differentiate into cell-cycle arrested stumpy forms when approaching a critical parasite density. Afterwards, the number of circulating parasites declines due to the immune response of the host against the Variant Surface Glycoprotein (VSG) coat expressed by the parasites and senescence of stumpy forms. A new wave emerges when a slender population expressing a new VSG arises. Differentiation of slender forms into stumpy forms is triggered by a density-dependent mechanism signaled by a parasite-derived molecule that is secreted by the parasites and accumulates as parasitemia rises. Adapted from 15.

1.3. Hallmarks of Stumpy forms

Besides showing clear morphological differences when compared to slender forms, stumpy forms also exhibit other characteristics that allow them to fulfill their transmission potential. One of the most recognizable hallmarks of stumpy forms is that they are uniformly arrested in G1/G0 phases of their cell-cycle, as opposed to slender forms that proliferate unchecked. This ensures that organelle repositioning occurring when BSFs progress to PCFs in the insect vector is coordinated with re-entry into cell division[16].

The midgut of the tsetse fly represents a challenging environment for parasites due to several reasons: acidic pH, decrease in temperature and proteolytic stress. Stumpy forms are prepared to tolerate such conditions, being their resistance to mild acid conditions and proteolytic stress used as an assay to monitor differentiation to transmissible forms[17]. While the blood provides an abundant source of glucose, enabling BSFs to generate ATP exclusively from glycolysis, deprivation of glucose in the midgut of the insect vector implies a metabolic switch for utilization of aminoacids, mainly proline[10,18]. Accordingly, stumpy forms are pre-adapted for such transformation, as demonstrated by development of a more elaborate mitochondrion with activation of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase from the tricarboxylic acid (TCA) cycle and a functional NADH dehydrogenase from the complex I of the respiratory chain[19,20]. These evidences have validated the use of dyes specific for mitochondria as tools for identifying stumpy forms, as these exhibit large cristae in the mitochondrion[21].

Rapid and synchronous differentiation to PCFs is probably one of the most important attributes of transmissible stumpy forms. A decrease in temperature (from 37 °C to 27 °C) and supplementation of the culture medium with TCA intermediates, namely citrate and/or cis-aconitate, is sufficient to trigger differentiation of BSFs into PCFs *in vitro* by stimulating this metabolic pathway and leading to the subsequent changes in morphology and gene expression that characterize this transition[22,23].

Changes in gene expression are essential for stumpy cells to survive in the midgut of the tsetse fly and to synchronously differentiate to PCFs upon receiving the appropriate stimuli. A carboxylate-transporter family, named Proteins Associated with Differentiation (PAD), were identified as key molecular components required for transmission of the cis-aconitate and citrate differentiation signals[24]. PAD1 is present on the surface of stumpy cells and its expression correlates strongly with capacity of BSFs to progress to PCFs. As PAD1 expression is absent in slender forms, it is a reliable and widely used molecular marker for identifying and quantifying stumpy forms. On the contrary, PAD2 is enriched in PCFs comparing to stumpy forms, being upregulated and changing its location from the flagellar pocket to the surface of the cell upon

reaching 20 °C, which is consistent with the temperature drop *in vivo* that occurs upon ingestion by the insect vector[25]. This might constitute a vital mechanism to inhibit premature differentiation of stumpy forms to PCFs in the bloodstream.

In contrast with stumpy form parasites, slender form parasites can change their surface coat to evade host immunity by expressing a different VSG at a time from a wide repertoire of genes that altogether are located to about 25 telomeric expression sites[26] (ES). Within each ES, several Expression-Site-Associated Genes (ESAG) are co-transcribed with the VSG gene, being ESAG9 family developmentally regulated in BSFs, as these genes are only expressed when parasites progress to stumpy forms. Moreover, these transcripts are some of the most upregulated during early stumpy formation *in vivo*[27], serving also as molecular markers for monitoring this transition.

1.4. Stumpy induction factor (SIF)

Differentiation of slender forms to stumpy forms is an irreversible process mediated by a density-sensing mechanism, as demonstrated by growth arrest and progression of replicative slender forms to non-dividing stumpy forms in *vitro*, when reaching critical parasite numbers in culture[28]. Experiments using conditioned medium collected from BSF cultures at maximal parasite density and supplemented to pleomorphic cultures resulted in growth arrest, increase of NADH dehydrogenase activity and morphological transformation to transmissible forms[12]. These evidences suggested the release of a SIF, a parasite-derived molecule that accumulated in conditioned medium as cell density increased, activating a signaling pathway that triggers cell-cycle arrest and production of stumpy forms. Although elusive, it has been established that SIF is a soluble compound with a molecular mass lower than 500 Da, as suggested by maintenance of inducing activity after ultrafiltration of conditioned medium with a nominal cutoff of 500Da[12]. Additionally, this compound was found to be heat stable (15 minutes at 90°C), making it unlikely for SIF to be a protein[12].

Interestingly, *T. brucei* laboratory-derived strains that were selected after several passages in mice for continuous proliferation in the bloodstream without fully differentiating to the tsetse fly infective form – termed monomorphic strains[29] – are capable of producing and releasing SIF into the conditioned medium, despite being non-responsive to this compound[12]. This suggests that monomorphic cell lines might be missing the required molecular components for receiving or/and transmitting the *quorum sensing* signal intracellularly.

So far, the unknown identity of SIF has made it difficult to characterize the molecular cascade involved in the transduction of the *quorum sensing* signal. However, some compounds

reproducing the function of SIF *in vitro* have been described that have elucidated the potential involvement of the cyclic AMP (cAMP) signaling pathway[12]. During infection by pleomorphic *T. brucei*, it was observed that the intracellular content of cAMP increased up to three-fold as parasitemia reached a peak, and declined while slender forms differentiated into intermediate and stumpy forms[30]. Strikingly, levels of this nucleotide in parasites from monomorphic strains were relatively low and did not suffer any oscillations throughout infection, supporting the role of cAMP in triggering a developmental switch to transmissible forms. Following these observations, the inducing effect of a cell-permeable cAMP derivative 8-(4-chlorophenylthio)-cAMP (8-pCPT-cAMP) was tested in pleomorphic strains and compared to the effect of SIF naturally present in conditioned medium[12]. 8-pCPTcAMP promoted cell-cycle arrest and differentiation of BSFs similarly to SIF, through a mechanism that involved activation of the cAMP pathway. Recent studies showed that the hydrolysis products of AMP analogues are accountable for the growth-inhibiting activity, as validated by the incapacity of hydrolysis-resistant cAMP derivatives to promote slender-to-differentiation to stumpy forms *in vitro*[31].

Treatment of parasites with troglitazone, an antidiabetic which exerts its effect on glucose metabolism and fatty acid utilization, has also been shown to promote slender to stumpy differentiation in pleomorphic strains, as evidenced by morphological transformations[32]. Moreover, monomorphic parasites exposed to this compound of the thiazolidinedione family became cell-cycle arrested and also exhibited activation of mitochondrial enzymes which are characteristic of stumpy forms. Ultimately, troglitazone-treated BSFs also transformed into PCFs with a greater efficiency compared to control cells upon receiving the appropriate stimuli[32].

1.5. Glucose metabolism in *T. brucei* BSFs

During the mammalian stage of its life cycle, *T. brucei* exhibits a repressed mitochondrial function, depending completely on glycolysis to fulfill its energy demands by converting glucose mainly into pyruvate under aerobic conditions. Due to lack of a functional TCA cycle and an absent respiratory chain[33], pyruvate is not further metabolized and is consequently secreted from the cell into the bloodstream. Compartmentalisation of this metabolic pathway within a specialized organelle named glycosome contrasts with other eukaryotic organisms in which glycolysis is mainly cytosolic[34]. The first seven enzymes of glycolysis that catalyze the conversion of glucose into 3-phosphoglycerate and two enzymes involved in the glycerol metabolism are sequestered in this peroxisome-like organelle, preventing turbo-explosion of glycolysis that would result from lack of feedback regulation of the first enzymes[35]. Net production of 2 ATP molecules per molecule

of glucose takes place in the cytosol through the reaction of pyruvate kinase (PYK). The physical separation of most of the glycolytic pathway from the cytosol avoids accumulation of intermediates, like glycerol-3-phosphate, that would otherwise be toxic to the parasites[35].

ATP and reducing equivalents production and consumption in the glycosome are balanced: ATP is consumed by the activity of hexokinase (HXK) and phosphofructokinase (PFK), while it is regained by the reaction catalyzed by the glycosomal phosphoglycerate kinase (PGK). Additionally, NADH generated through the reaction of glyceraldehyde-3-phosphate dehydrogenase is balanced when re-oxidized inside the glycosome upon transformation of dihydroxyacetone phosphate (DHAP) into glycerol-3-phosphate (Gly-3-P). The latter metabolite is shuttled to the mitochondrion where it provides electrons to O_2 and is reconverted to DHAP, in a process that involves the Trypanosome Alternative Oxidase (TAO). Although cyanide insensitive, TAO can be inhibited by salicylhydroxamic acid (SHAM), mimicking anaerobic conditions. This treatment alone does not kill trypanosomes, requiring the addition of glycerol to exert a toxic effect[36], since this metabolite is a reported inhibitor of anaerobic glycolysis in *T. brucei*[37]. Parasites can thrive in anaerobic conditions by metabolizing glucose into equal amounts of pyruvate and glycerol, despite the decrease of the ATP yield by 50%. Gly-3-P that is formed in the glycosome is not oxidized into DHAP in the mitochondrion and consequently accumulates, leading to the reversal of the reaction of glycerol kinase (GK)[38,39]. As a result, glycerol and ATP are produced in a reaction that is normally thermodynamically unfavorable, ensuring that the ATP/ADP balance is maintained even in the absence of oxygen. Nonetheless, it was described that even under aerobic conditions glucose is not completely metabolized into pyruvate, and 10% of the end-products of glycolysis is glycerol[40].

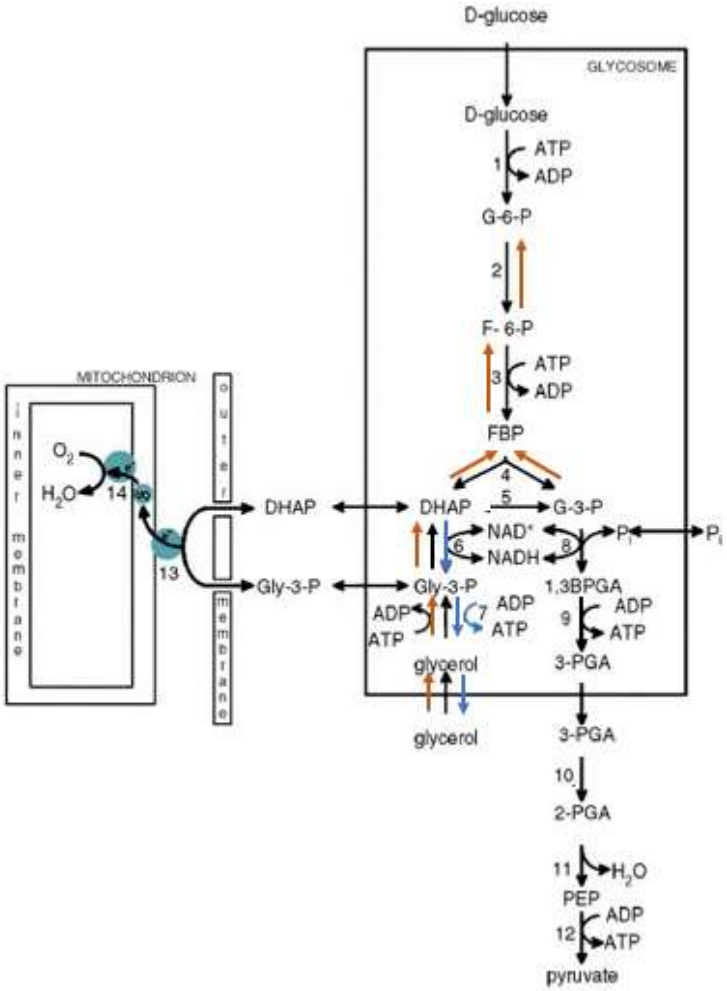

Figure 1.3 - Schematic representation of glycolysis in *T. brucei* BSFs. Under aerobic conditions, glucose is almost completely converted into pyruvate. However, under anaerobic conditions, equimolar amounts of pyruvate and glycerol are produced. Blue arrows represent reactions that occur mainly under anaerobic conditions and orange arrows represent reactions that occur in glucose-depleted environments. Adapted from 36 and 41. Abbreviations: 1,3BPGA, 1,3-bisphosphoglycerate; DHAP, dihydroxyacetone phosphate; F-6-P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; G-3-P, glyceraldehyde 3-phosphate; G-6-P, glucose 6-phosphate; Gly-3-P, glycerol 3-phosphate; PEP, phosphoenolpyruvate; 3-PGA, 3-phosphoglycerate; Pi, inorganic phosphate; UQ, ubiquinone pool. Enzymes are: 1, hexokinase: 2, glucose-6-phosphate isomerase; 3, phosphofructokinase; 4, aldolase; 5, triosephosphate isomerase; 6, glycerol-3-phosphate dehydrogenase; 7, glycerol kinase; 8, glyceraldehyde-3-phosphate dehydrogenase; 9, glycosomal phosphoglycerate kinase; 10, phosphoglycerate mutase; 11, enolase; 12, pyruvate kinase; 13, FAD-dependent glycerol-3-phosphate dehydrogenase; 14, alternative oxidase.

1.6. Transport of glycerol in *T. brucei*

For BSFs to survive in oxygen-depleted environments, Gly-3-P is converted into glycerol with concomitant production of ATP via an alternative pathway of glycolysis. To fuel this thermodynamically highly unfavorable reaction, glycerol needs to be rapidly removed from the cytosol in order to drive the reverse reaction of glycerol kinase through accumulation of high concentrations of Gly-3-P inside the glycosome[42]. Accordingly, when combining the presence of SHAM – an inhibitor of aerobic glycolysis – with the addition of 5mM of glycerol to the culture medium, parasites die rapidly due to inhibition of ATP synthesis[43]. Although transport of glycerol across the plasma membrane can occur through simple diffusion, this process is mainly mediated by small protein channels termed aquaglyceroporins (AQPs) that also facilitate transport of water and other uncharged molecules, such as urea, ammonia, dihydroxyacetone, and even arsenite[44]. Three AQPs have been identified in *T. brucei* that differ mostly in their subcellular localization and

gene regulation throughout the life cycle of the parasite[45]. TbAQP1 is localized to the flagellum and is nearly the only AQP securing facilitated diffusion in PCFs, TbAQP3 is only found in the plasma membrane of BSFs, being abundantly expressed in replicative slender forms. On the other hand, TbAQP2 was slightly expressed in both the insect stage and the mammalian stage of the life cycle, sequestering to the flagellar pocket in BSFs.

TbAQP2 plays an important role in mediating drug uptake in *T. brucei*, making parasites sensitive to the trypanocidal action of melarsoprol and also pentamidine[46]. The former is an arsenic derivative widely used as treatment for the late-stage of both *T. b. gambiense* and *T. b. rhodesiense* infections. Indeed, knockout of TbAQP2 in parasites leads to melarsoprol and pentamidine cross-resistance *in vitro* and also enhances parasite sensitivity to respiratory inhibitors, such as TAO, even in the absence of exogenous glycerol[47]. The latter evidence reinforces the function of TbAQP2 as the major contributor to glycerol efflux across the membrane. Deletion of all three AQPs in *T. brucei* was already performed without resulting in any obvious phenotype regarding viability and osmoregulation, proving that these transporters are non-essential to parasites. However, considerable defects in glycerol uptake and utilization were observed in triple-null cells, consistent with negligible simple diffusion of this metabolite across the plasma membrane[47]. Overall, AQPs have a relevant physiological function in glycerol metabolism, allowing *T. brucei* to maintain energy production even under aerobic conditions.

2. Work aims

It was recently observed in our laboratory that glycerol can promote prolonged growth arrest in *T. brucei* cultures and also earlier parasite differentiation to quiescent stumpy forms, making this compound a good candidate for a Stumpy induction factor (SIF). My main aim was to elucidate if in fact this metabolite is excreted by parasites and accumulates extracellularly according to parasite density, driving differentiation of slender forms to stumpy forms. Therefore, to fulfil our aim, this project was divided into two main parts:

(1) – Dissect whether the presence of glycerol in the culture medium is directly correlated with differentiation into stumpy forms;

(2) – Test the role of glycerol in differentiation, by employing genetic tools that abrogate either glycerol metabolism or glycerol uptake and efflux in parasites.

3. Materials and methods

3.1. Parasite lines and parasite culture

Most of the experiments were performed using a parasite line derived from the pleomorphic EATRO 1125 AnTat 1.1 90:13 parasite line that contains both pLew90 and pLew13 constructs, enabling co-expression of T7 RNA Polymerase and a tetracycline repressor[48]. In this particular case, the EATRO 1125 AnTat 1.1 90:13 GFP::PAD1 stumpy reporter parasite line was used, consisting of a cell line in which expression of a green fluorescent protein (GFP) is under regulation of the 3'UTR of PAD1[49], leading to a GFP signal that concentrates to the nucleus upon parasite differentiation to stumpy forms. Additionally, the monomorphic cell line Lister 427 PL1A[50] GFP::PAD1, isogenic of Lister 427 PL1S (a cell line described by Yang *et al.*[51]), was also used in the experiments performed with conditioned medium to assess if SIF would have an effect on the growth of parasites that are only capable of differentiating to stumpy-intermediate forms. *T. brucei* BSF parasites were cultured in HMI-11 medium (with an identical composition to HMI-9, but lacking Serum plus[52]) at constant temperature of 37°C and with a 5% content of CO_2 in the air. Parasites were subcultured nearly every day by diluting the cells in fresh medium in order to prevent cell density from reaching $5x10^5$ parasites/mL. To further clarify the role of glycerol in inducing differentiation of slender forms to stumpy forms *in vitro*, two parasite mutant cell lines were generated that were deficient for glycerol production and glycerol mediated transport: AnTat 1.1 90:13 GFP::PAD1 GK-RNAi and AnTat 1.1 GFP::PAD1 Δaqp1-3/Δaqp1-3, respectively.

3.2. *In vitro* differentiation of slender forms to stumpy forms

To test the effect of glycerol in promoting slender to differentiation to stumpy forms *in vitro*, AnTat 1.1 90:13 GFP::PAD1 parasites were cultured in HMI-11 medium supplemented with glycerol (Sigma-Aldrich) for 3 days without passage. In most of the experiments, glycerol was added to the culture medium at a concentration of 68mM by preparing a pre-dilution of this compound at 10% (v/v) with HMI-11 medium and filtering this solution through a membrane with a 0,22µm pore size (Millipore) to avoid contamination. To establish AnTat 1.1 90:13 GFP::PAD1 cultures in the presence of exogenous glycerol, the previous solution was then mixed to fresh HMI-11 at a dilution of 1:20. For comparison, experiments were also performed in which AnTat 1.1 90:13 GFP::PAD1 parasites were supplemented with 8-pCPT-AMP 100µM (Sigma-Aldrich) and sodium pyruvate 6mM (Sigma-Aldrich) in the culture medium. The proportion of stumpy forms generated *in vitro* was determined daily by flow cytometry through quantification of GFP-PAD1 expressing parasites. Additionally, parasite density in culture was assessed daily by counting cells in a Neubauer chamber.

3.3. Conditioned medium experiments

Conditioned media of the pleomorphic parasite line EATRO 1125 Antat 1.1 and the monomorphic parasite line Lister 427 PL1A GFP::PAD1 were prepared by initiating cultures with a density of $1x10^5$ cell/mL and collecting the culture medium when reaching maximal density after 2 days (approximately $3x10^6$ cell/mL). For that purpose, both cultures were centrifuged at 1800 rpm for 10 minutes and the supernatant was filtered through a membrane with a 0,22µm pore size (Millipore). AnTat 1.1 90:13 GFP::PAD1 and Lister 427 PL1A GFP::PAD1 cultures were then established using equal volumes of conditioned medium and fresh HMI-11 to evaluate the effect on differentiation to stumpy forms.

3.4. Procyclic transformation *in vitro*

To induce transformation of BSFs to PCFs in vitro, 5 million parasites were transferred to Differentiation Trypanosome Medium (DTM) containing cis-aconitate (Sigma-Aldrich) 6mM at a density of $1x10^6$ parasites/mL. Cultures were incubated at a temperature of 27°C and with 5% CO_2 content in the air. To assess the efficiency of this transformation, the amount of parasites expressing procyclin on their surface was quantified, after 18h and 48h of incubation, by flow cytometry using an anti-procyclin antibody (Cedarlane Laboratories) at a dilution of 1:500.

3.5. Flow cytometry

500 µL of each culture were collected and parasites were pelleted by centrifugation at 10 000 RCF for 2 minutes, the supernatant was removed and cells were resuspendend in 500 µL of TDB (5mM KCl, 80mM NaCl, 1 mM $MgSO_4$, 20mM Na_2HPO_4, 2mM NaH_2PO_4, 20mM glucose, pH 7.4). For PCF cultures, centrifugations were performed at 4°C and samples were kept in ice. Flow cytometry analysis was performed in BD Accuri C6 (BD Biosciences) and data was analyzed using the software FlowJo X version 10.0.7r2.

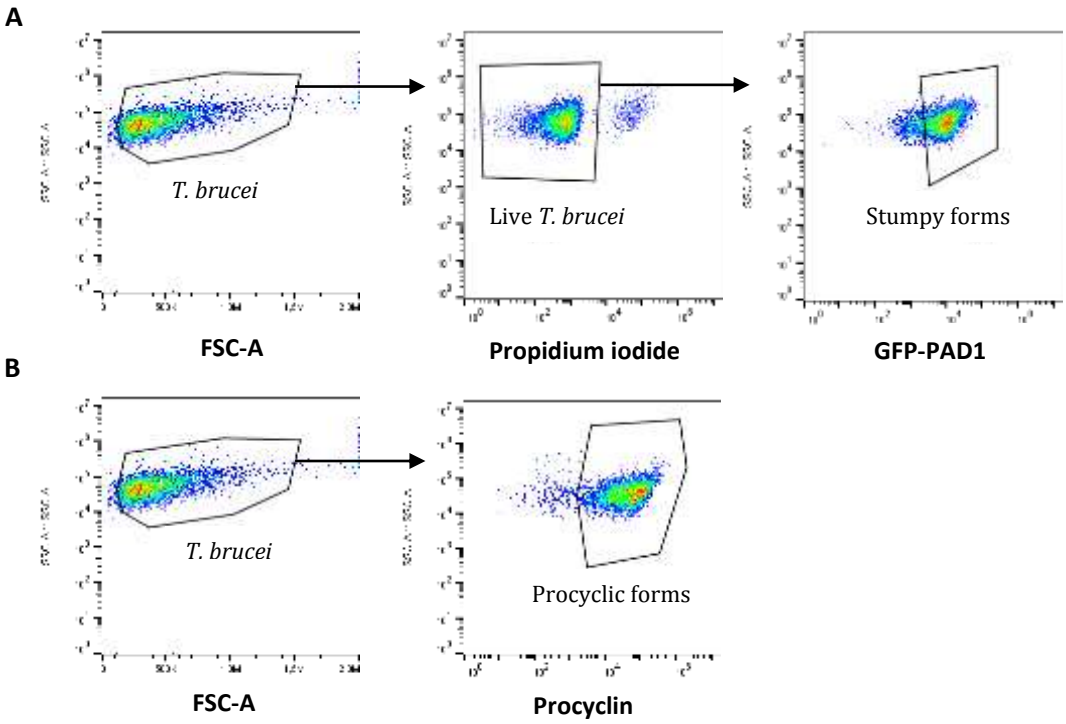

Figure 3.1 – General gating strategy adopted in flow cytometry. (A) *T. brucei* bloodstream stumpy forms. (B) *T. brucei* insect procyclic forms

3.6. Glycerol and pyruvate quantification

Glycerol was quantified in conditioned medium collected from BSF cultures and also in the serum of infected mice using the Glycerol Assay Kit (Sigma-Aldrich) according to the instructions of the manufacturer. Pyruvate was also quantified in conditioned medium collected from BSF cultures using the Pyruvate Assay Kit (Sigma-Aldrich) according to the instructions of the manufacturer.

3.7. Generation of mutant parasite lines

3.7.1. General cloning strategy

In this project, a traditional cloning strategy was employed that consisted in generating the DNA inserts by PCR amplification from the *T. brucei* AnTat 1.1 90:13 genome using the Phusion High-Fidelity DNA Polymerase (Thermo Scientific), followed by digestion of the inserts and the plasmids with the appropriate restriction enzymes to generate complementary ends. The DNA fragments were then purified using the MinElute PCR Purification Kit (Qiagen) and joined to a vector using T4 DNA ligase (New England Biolabs) in a reaction performed overnight at 14°C. *Escherichia coli* (*E. coli*) competent cells were then transformed with the ligation products for amplification of the plasmids. Positive colonies were selected by culture in plates containing LB medium with ampicillin 100 μg/mL and the plasmids were purified using the NZYMiniprep (NZYTech) according to the instructions of the manufacturer. The extracted DNA was then tested

for the presence of the constructs for gene deletion or depletion by restriction digestion followed by electrophoresis of the obtained DNA fragments.

3.7.2. Constructs for deletion of AQP1-3

TbAQP1 (Tb927.6.1520, TriTrypDB) is localized to chromosome 6 and TbAQP2 (Tb927.10.14170, TriTrypDB) and TbAQP3 (Tb927.10.14160, TriTrypDB) are adjacent to each other on chromosome 10. Consequently, we deleted simultaneously the AQP2 and AQP3 genes, followed by deletion of the AQP1 gene, via replacement with drug resistance cassettes by homologous recombination in AnTat 1.1. GFP::PAD1 parasite line. Since the genome of *T. brucei* is diploid, two knockout constructs containing different resistance markers were created to target each locus (AQP2-3 locus and AQP1 locus) in both alleles. For that purpose, we generated four DNA fragments with approximately 200 base pairs (bp) each, by amplification of part of the regions flanking both the AQP2-3 and the AQP1 coding sequences (CDS), termed homologous regions (HR). Afterwards, the fragments of both the AQP2-3 and AQP1 HRs located at the 5' end (5'HR), were digested with KpnI and MluI enzymes (New England Biolabs) and cloned into plasmids containing the Neomycin phosphotransferase gene (NEO) and the puromycin N-acetyl-tranferase gene (PAC), respectively. Two of the required constructs for AQP deletion (pAT7 and pAT9) were obtained by digesting the 3'HR fragments of AQP2-3 and AQP1 with SmaI and SacI (New England Biolabs) and inserting them in the respective vectors. Finally, for replacement of the genes in both alleles, two other plasmids analogous to pAT7 and pAT9 (pAT8 and pAT10, respectively) were created by replacing the resistance cassettes with hygromycin phosphotransferase (HYG) and phleomycin hydrolase (PHLEO) genes, respectively. For that intent, HYG and PHLEO fragments were digested with MluI/SmaI and BamHI/HindIII (New England Biolabs), respectively. The resistance cassettes were cloned into the respective vectors via T4 DNA ligase reaction. Before transfection of *T. brucei* BSFs, all four plasmids were linearized with the restriction enzymes KpnI and SacI and the DNA fragments were purified by running agarose gels and excising the smaller band, followed by purification using the NZYGelpure kit (NZYTech).

3.7.3. Constructs for GK RNAi-mediated depletion

Two PCR products were generated with a fragment of 400 bp amplified from the CDS of GK (Tb927.9.12550) followed by a random sequence of 50 bp. Both fragments had an EcoRI restriction site at the 3' end, differing only in the restriction sites present upstream of the gene-specific region (BamHI and HindIII). PCR products were digested with 140U of EcoRI in 0,2 mL for 2,5 hours and purified using the MinElute PCR Purification Kit (Qiagen). The digested products were then ligated with 1000U of T4 DNA ligase (New England Biolabs) at 16 ºC for 16 hours to produce the inverted repeats that were later purified by gel extraction using the NZYGelpure kit

(NZYTech). The inverted repeats and the pFMG10 plasmid were digested with BamHi and HindIII to and ligated together. The resulting plasmid was digested with NotI (New England Biolabs) and the fragment was purified by gel extraction using the NZYGelpure kit.

3.7.4. Bacteria transformation

25 µL of JM109 competent *E. coli* cells (Promega) were transformed with 10 µL of the products of the ligation reactions, followed by incubation on ice for 30 min and heat shock at 42 ºC. Afterwards, cells were incubated for 1-2 minutes on ice and 900 µL of LB medium were added to the tubes. The cell suspension was agitated for at least 1 hour at 37 ºC and subsequently, transformed cells were plated at 1:1 and 1:10 dilutions in selective medium containing 100 µg/mL of ampicillin and incubated overnight at 37 ºC. The following day, single colonies were inoculated into LB medium containing 0,1% ampicillin and grown overnight.

3.7.5. Transfection of parasites with DNA constructs

For delivery of the DNA constructs to the parasites, 12-25 million cells were pelleted by centrifugation at 1800 rpm for 10 minutes and the culture medium discarded. The cells were resuspended and transferred to a tube that was centrifuged in an Eppendorf MiniSpin microcentrifuge (Eppendorf) at 13400 rpm for 30 seconds in order to remove the remaining supernatant. The parasites were resuspended in 100 µl of fresh Tb-BSF buffer[53] (90 mM $NaPO_4$, 5 mM KCl, 50 mM HEPES, 0.15 mM $CaCl_2$, pH7.3) and 2-3 µg of the linearized constructs, transferred to a Gene Pulser/MicroPulser Electroporation cuvette (Bio-Rad) and transfected with the AMAXA Nucleofector II (Lonza) using the program X-001. Parasites were immediately transferred to 30 mL of warm HMI-11 medium containing the selective drugs for the parental cell line and plated in 24-well plates at 1:1, 1:10 and 1:100 dilutions. The following day, the appropriate selective drugs were added to the culture medium. Approximately 6 days after the transfection, positively selected clones reach considerable numbers that allow expansion in fresh culture medium to perform both DNA and RNA extraction to validate the mutations.

3.7.6. Genomic DNA (gDNA) extraction and PCR genotyping

To confirm that the DNA constructs integrated in the correct loci, genomic DNA was extracted from the mutant parasite lines and their respective parental parasite lines by centrifuging the cell suspensions at 1800 rpm for 10 minutes and resuspending in 0,5 mL of DNAzol (Invitrogen) by pipetting up and down repeatedly. After incubation of the samples for 5 minutes at RT, 0,25 mL of ethanol 100% was added. Tubes were inverted 5-8 times to promote precipitation of gDNA and incubated again for 5 minutes at RT. DNA was pelleted by centrifuging the tubes at maximal speed for 15 minutes, followed by removal of the supernatant. Then, gDNA was washed two times with ethanol 70% by centrifuging at maximal speed for 5 minutes and discarding the supernatant.

Finally, the remaining supernatant was removed by centrifuging at maximal speed for 1 minute. DNA was quickly resuspended in 20-30 µL of EB buffer from the MinElute PCR Purification Kit (Qiagen).PCR reactions were assembled by adding 50-200 ng of each gDNA sample, 10 µL of Phusion HF Buffer (Thermo Scientific), 0,5 µL of Phusion High-Fidelity DNA Polymerase (Thermo Scientific), 1 µL of dNTPs 10mM (Sigma Aldrich), 0,5 µL of the forward primer, 0,5 µL of the reverse primer, and water to adjust the volume to 50 µL. Afterwards, the PCR products were run on 1% agarose (NZYTech) gels stained with Gel Red (VWR) for approximately 50 minutes at 100V. Agarose gels were then visualized on ChemiDoc XRS+ (Bio-Rad).

3.8. Total RNA extraction and quantitative PCR (qPCR)

Total RNA was extracted from at least 10 million parasites from cultures with a density below $5x10^5$ parasites/mL. Cells were pelleted by centrifugation (at 4°C, 10000 RCF, 2 minutes) to discard the culture medium and were lysed in 1mL of TRIzol (Invitrogen, Life Technologies) by pipetting up and down repeatedly and incubating for 5 minutes at room temperature (RT). RNA extraction was performed according to the instructions of the manufacturer. DNase treatment was performed to ensure that the samples were not contaminated with genomic DNA. For that purpose, 44 µL of each RNA sample were incubated with 1 µL of DNase I (New England Biolabs) and 5 µL of DNase I buffer (New England Biolabs) at 37°C for at least 30 minutes. Afterwards 0,5 µL of EDTA 0,5M (Ambion) were added and the reaction was inactivated at 75°C for 10 minutes. The concentration of RNA in each sample was determined previously and also after the DNase treatment. 240 ng of RNA from each sample were converted to cDNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies) using random primers and a Thermal Cycler (Bio-Rad) programmed for the required temperatures and times of incubation. The qPCR reactions were assembled in a MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems) by adding 4 µL of each cDNA sample (previously diluted 10 times), 1 µL of the primers targeting the gene of interest and 5 µL of the SYBR Green PCR Master Mix (Applied Biosystems). The plate was centrifuged at 2000 rpm for 2 minutes and analysed on StepOnePlus Real-Time PCR System (Applied Biosystems).

3.9. Western blotting

Total protein extracts were prepared by pelleting parasites through centrifugation at 10000 RCF for 2 minutes and resuspending in 1 volume of Laemmli sample buffer (2.4ml of 1M Tris HCl pH 6.8, 0.8g of SDS, 4ml 100% glycerol, 1ml of bromophenol blue (0.5%), 1ml of β-mercaptoethanol and 2.8 ml water) and 3 volumes of RIPA buffer (50mM TrisHCl pH8, 150mM

NaCl, 1% NP_40, 0.5% Sodium Deoxycholate and 0.1% SDS) to a final density of $1x10^6$ cells per 10 µL of Laemmli sample buffer 1X. Samples were then boiled for 10 minutes at 100°C and 10 µL of each sample , as well as 5 µL of Precision Plus Protein Dual Xtra standard ladder (Bio-Rad), were loaded into separate wells in Any KD Mini-Protean TGX precast gels (Bio-Rad). Electrophoresis was performed at 100V with 1 volume of Tris-glycine running buffer 10X (30g Tris base 0,25M, 144g Glycine 1,92M, 100ml SDS 10% and adjusting with ddH2O until 1L of volume) and 9 volumes of ddH_2O, during 1 hour and 20 minutes. The gels were washed in ddH2O and the separated proteins were transferred into nitrocellulose membranes using an iBlot Dry Blotting System (Invitrogen). Membranes were incubated in a blocking solution containing TBS 1X (5 mM Tris-HCl pH 8.0 and 15 mM NaCl), milk powder 5% (m/v) and Tween-20 0,1% (v/v) for at least 1 hour with agitation. After three washes with TBS 1X/0,1% Tween-20, membranes were incubated in blocking solution containing the primary antibodies against glycerol kinase (1:1000 dilution, antibody kindly provided by Dr. Frédéric Bringaud) and histone H2A (1:7000 dilution, anti-rabbit, Pineda Antikörper-Service), overnight at 4°C. The following day, membranes were washed in TBS 1X/0,1% Tween-20 three times, followed by incubation with blocking solution containing the secondary antibody ECL Rabbit IgG conjugated with Horse Radish Peroxidase (HRP) (1:10000 dilution, GE Healthcare) for 1 hour at RT. Once again, membranes were washed 3 times in TBS 1X/0,1% Tween-20 and incubated with 2 mL of Western Lightning Plus-ECL solution (PerkingElmer) for 2-5 minutes. Membranes were exposed on ChemiDoc XRS+ (Bio-Rad).

3.10. Mice infection and parasitemia quantification

In vivo infections by *T. brucei* were carried in 10 to 18 week-old, wild-type male C57BL/6J mice from Charles River Laboratories International. Mice were maintained at the Rodent Facility of the Instituto de Medicina Molecular, under pathogen-free conditions, with a temperature between 21°C and 22°C, and a 12h light/12h dark cycle. Each group was housed in individually ventilated cage and fresh water and food were available ad libitum. All the experimental work in animals was performed in agreement with the EU regulations and was approved by the Animal Care and Ethical Committee of iMM (AWB_2016_07_LF_Tropism). One group of C57BL/6J mice (4 animals each) was infected with 2000 AnTat 1.1 90:13 GFP::PAD1 parasites, while two other groups were infected with 2000 AnTat 1.1 90:13 GFP::PAD1 GK RNAi parasites by intraperitoneal injection. One of the mice groups infected with AnTat 1.1 90:13 GK RNAi parasites was supplemented with doxycycline hyclate 50 mg/mL (Sigma-Aldrich) in their drinking water every two days, with the purpose of inducing the RNAi machinery. Parasitemias were determined at selected time points during 58 days through collection of 1 µL of blood from the tail vein and manually counting the number of parasites in a Neubauer chamber.

Table 3.1 - List of oligonucleotides designed to amplify the DNA inserts required for creating the DNA constructs. All oligonucleotides were designed using the SeqBuilder software (DNASTAR) and ordered to Sigma-Aldrich. Gene-specific regions are represented with uppercase letters, and underline lowercase represents the restriction sites for the enzymes. A randomized segment of 50 nucleotides is represented by $(N)_{50}$.

Primer sequence	Description
tatagggcgaatt<u>gggtacc</u>TGGTGGAGAGATATTTACAACGG	Sense primer to amplify 5'HR of AQP2-3
atgtaaggaa<u>acgcgt</u>TTACACAAATAGTCAGCAGCGG	Anti-sense primer to amplify 5'HR of AQP2-3
tatagggcgaatt<u>gggtacc</u>CGTAGCATAATCGTGTCCC	Sense primer to amplify 5'HR of AQP1
atgtaaggaa<u>acgcgt</u>TATTTACCGTACCATAACCAATG	Anti-sense primer to amplify 5'HR of AQP1
gggggattcgag<u>cccggg</u>CTGCATGAAGGAAAAAGGAAG	Sense primer to amplify 3'HR of AQP2-3
gggaacaaaagct<u>ggagctc</u>GGTTCAGCGCTATTCAGTGAC	Anti-sense primer to amplify 3'HR of AQP2-3
gggggattcgag<u>cccggg</u>GGGGGAAAAGAACAAGAC	Sense primer to amplify 3'HR of AQP1
gggaacaaaagct<u>ggagctc</u>AAACATCGTTTCGAACATTTG	Anti-sense primer to amplify 3'HR of AQP1
acggtaaata<u>acgcgt</u>AAGCTAGGGGCACAGCAAGG	Sense primer to amplify HYG CDS
ttcttttccccc<u>cccggg</u>GGGGCTCGAATCCCCCCA	Anti-sense primer to amplify HYG CDS
ccc<u>aagctt</u>ATGGCCAAGTTGACCAGTGC	Sense primer to amplify PHLEO CDS
cgc<u>ggatcc</u>TCAGTCCTGCTCCTCGGC	Anti-sense primer to amplify PHLEO CDS
gttccc<u>aagctt</u>tctagaCAAAGCTGAGGCAAAAGGAC	Sense primer to amplify GK CDS
ggttcgc<u>ggatcc</u>tctagaCAAAGCTGAGGCAAAAGGAC	Sense primer to amplify GK CDS
<u>ggaattc</u>ctgtaggcacNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNATCCATAAGGAAGGTGCGTG	Anti-sense primer to amplify GK CDS

Table 3.2- List of oligonucleotides designed to confirm correct integration of the DNA constructs and to assess gene expression by quantitative PCR (qPCR). All oligonucleotides were designed using the SeqBuilder software (DNASTAR) and ordered to Sigma-Aldrich.

Primer sequence	Description
CCAAAATCAGCGGGTTCAC	Sense primer to amplify AQP2-3 locus
TTAGGGGTGCAACGAGGG	Anti-sense primer to amplify AQP2-3 locus
GCGAATATTTGTGCTTGCC	Sense primer to amplify AQP1 locus
TTTGTTGCTGTTACTGTTGGTTAC	Anti-sense primer to amplify AQP1 locus
GGCATTGGCGAGGGTAAC	Sense primer to amplify part of AQP1 CDS
TCATGGGTAATGGTGTAGTGGC	Anti-sense primer to amplify part of AQP1 CDS
GTTTCACTGGGCATCTCCG	Sense primer to amplify part of AQP2 CDS
CAGCAAACACTCCGTAAGCG	Anti-sense primer to amplify part of AQP2 CDS
AGAAAAGTCCCCGGCTACATC	Sense primer to amplify part of AQP3 CDS
GCAATCAACTCACCACCACC	Anti-sense primer to amplify part of AQP3 CDS
TGCGCACGTACGACATCAC	Sense primer to amplify part of GK CDS
CATTCTCGAGCATCCACCG	Anti-sense primer to amplify part of GK CDS

4. Results

4.1. Glycerol promotes differentiation of slender forms into stumpy forms *in vitro*

The hypothesis that glycerol could be a potential SIF is based on a previous observation made in our laboratory that this compound when used as a cryopreserving agent can trigger prolonged parasite growth arrest and premature differentiation of slender forms to stumpy forms if not removed upon thawing. Additionally, it seems plausible that glycerol could be one of the signaling molecules triggering differentiation via a density-dependent mechanism, since it is endogenous to *T. brucei* as one of the end-products of glycolysis, the sole metabolic pathway for ATP production in bloodstream forms (BSF). Consequently, since this compound is not further metabolized and is rapidly excreted by parasites, it would accumulate extracellularly as cell density increased.

Transition of slender forms to stumpy forms is characterized by several hallmarks that include cell-cycle arrest in G1/G0 phase[16], expression of specific genes that are essential for progression to the insect stage of the life-cycle, being PAD1 one of the most upregulated genes in transmissible forms[24,], and synchronous transition to PCFs[22,23]. In this section, we explore the effect of glycerol in promoting differentiation of *T. brucei* BSFs *in vitro* by assessing several molecular markers specific to stumpy forms. All the experiments were conducted using reporter parasite lines having expression of GFP under regulation of PAD1 3'UTR, allowing rapid and accurate quantification of stumpy forms within a parasite population through flow cytometry.

4.1.1. Preliminary experiments

Preliminary experiments *in vitro* have been performed in Luísa Figueiredo's lab in order to better understand the impact of glycerol on the differentiation of slender forms into stumpy forms of *T. brucei.* For this purpose, the effect of increasing concentrations of glycerol added to HMI-11 culture medium was tested on a pleomorphic cell line. The efficiency of parasite differentiation *in vitro* was monitored for 2 days by quantification of GFP-PAD1 expression. Additionally, cell-cycle profiling of parasites incubated with the highest concentration of glycerol tested (68mM) was also performed using flow cytometry to evaluate the percentage of parasites that were arrested in G1 phase (**Figure 4.1).** These results show that the percentage of stumpy forms in the parasite population is directly correlated with the initial concentration of glycerol supplemented to the culture medium (**Table 4.1**), reaching 24% and 65% of stumpy forms after 1 day and 2 days of incubation with the highest concentration of glycerol, respectively (**Figure 4.1A)**.The dose-dependent response of parasites to glycerol supports the potential of this metabolite as a SIF.

Moreover, cell cycle arrest in G1 phase is observed in the parasite population exposed to glycerol compared to the parasite population cultured in the absence of this compound (**Figure 4.1B)**. Indeed, while the percentage of non-induced parasites that are arrested in G1 phase does not increase between day 1 to day 2 of the experiment, the parasite culture incubated with exogenous glycerol shows an increase in cells arrested in G1 phase from 66% at day 1 to 75% at day 2. Therefore, glycerol promotes both the expression of the molecular marker PAD1 and G1 phase cell-cycle arrest, typical of stumpy cells, supporting the hypothesis that glycerol is a strong inducer of differentiation to stumpy forms of *T. brucei* parasites *in vitro*.

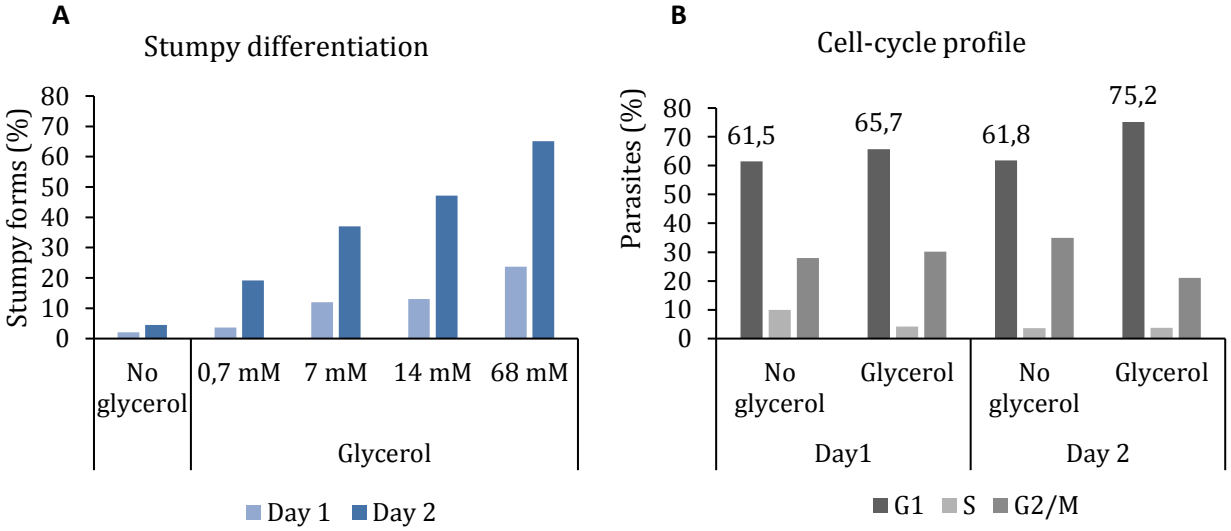

Figure 4.1- Glycerol promotes differentiation into stumpy forms and cell-cycle arrest of *T. brucei* parasites *in vitro*. (A) Percentage of stumpy forms in AnTat 1.1 GFP-PAD1 cultures incubated with different concentrations of glycerol (from 0,7mM to 68mM) determined by flow cytometry through quantification of GFP-expressing cells. (B) Cell-cycle profiling of AnTat 1.1 GFP-PAD1 parasites cultured in non-supplemented HMI-11 medium and HMI-11 medium supplemented with glycerol 68mM. Cell-cycle analysis of parasite population was performed using PI staining and measured by flow cytometry. (A, B) Initial parasite density in cultures was 10^5 parasites/mL. This experiment was performed one time with two independent biological replicates. These experiments were performed by Fabien Guegan.

Table 4.1 – Glycerol promotes *in vitro* differentiation of slender forms to stumpy forms in a dose-dependent manner. Fold change of percentage of insect-transmissible forms in AnTat 1.1 GFP-PAD1 *T. brucei* cultures induced with different concentrations of glycerol relative to the non-induced culture.

	[Glycerol] (mM)	0,7	7	14	68
Fold change of Sumpy forms	24 hours	1,8	5,9	6,4	11,6
	48 hours	4,3	8,3	10,5	14,5

4.1.2. Glycerol induces differentiation into fully functional stumpy forms

Besides morphologic changes and cell-cycle arrest, strong regulation of specific genes that are essential for surviving in the tsetse fly midgut or perceiving the differentiation signal to PCFs also accompanies the developmental switch of parasites from slender to stumpy forms. To monitor the impact of glycerol on stimulating differentiation, the transcript levels of genes specific or enriched in stumpy forms were determined in parasites treated with glycerol and compared to non-treated parasites using a qPCR approach. For this purpose, a *T. brucei* culture was maintained in two different conditions: one in which the culture medium was non-supplemented and another in which glycerol was added to the culture medium. Samples were collected at several timepoints (0, 6, 12, and 24 hours) in order to extract RNA and generate cDNA, followed by qPCR to quantify expression of genes relative to a "housekeeping" gene (**Figure 4.2**). Additionally, the extent of parasite differentiation was also determined by quantifying GFP-PAD1 expressing cells throughout the time course (**Table 4.2**). Interestingly, the effect of parasite density alone was not sufficient to promote differentiation of slender forms to stumpy forms, as demonstrated by a more or less constant proportion of tsetse-infective forms throughout the time course in cultures with plain HMI-11. However, in parasite cultures with HMI-11 containing glycerol, stumpy forms arise earlier comparing to the control condition, increasing gradually after only 12 hours of incubation.

PAD1 is one of the most widely used molecular markers for identifying stumpy cells, since its expression is negligible in slender forms and increases throughout the process of transition to stumpy forms[24]. Levels of PAD1 mRNA were shown to increase almost 6-fold after 24 hours of induction with glycerol, while in non-induced parasites upregulation of these transcripts was not shown by the same time point. A similar behavior was observed for the mRNA levels of GPEET procyclin, a class of procyclins that possesses a pentapeptide repeat and is expressed on the surface of early PCFs[54] that establish infection in the tsetse fly midgut. Despite being characteristic of the surface coat of PCFs, this molecule is positively regulated at the posttranscriptional level by glycerol, mediated by the GPEET 3'UTR[55], possibly explaining why glycerol-induced stumpy forms transform so rapidly into PCFs. ESAG9 (Expression site associated gene 9 family) are a group of genes that were reported as one of the most upregulated in transmissible forms, suffering also an increase in transcript levels upon parasite incubation with glycerol of approximately 2-fold, starting 12 hours post-incubation. The metabolic enzyme TAO is the only terminal oxidase in stumpy forms[56] ensuring maintenance of the redox balance, in addition to being previously described as enriched in stumpy forms[57]. After 24 hours, the transcript levels for this protein increased almost 4-fold in parasites comparing to initial levels, while parasites cultured in non-supplemented HMI-11 maintained expression of TAO more or less constant, as they still have not initiated the process of differentiating to fly-transmissible forms.

Overall, it is clear the rapid increase of expression of certain stumpy-marker genes in response to exogenous glycerol comparing to non-supplemented parasite cultures, being these evidences noticeable after only 12 hours of incubation. Therefore, the presence of glycerol triggers modifications in gene regulation that typically occur during transition of slender forms to stumpy forms, ensuring that parasites are prepared to invade the midgut of the insect vector.

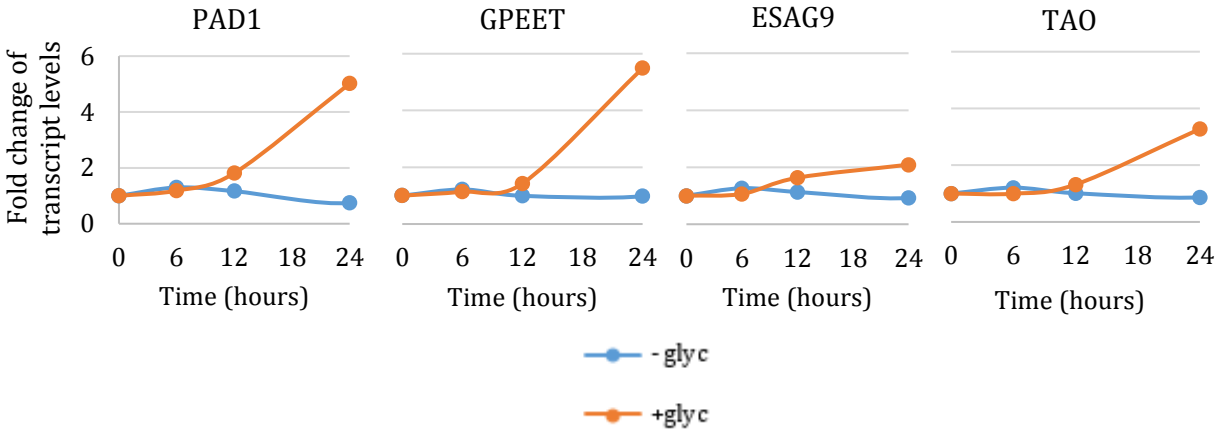

Figure 4.2 - Validation of the inducing effect of glycerol by upregulation of genes that are specific or related to differentiation to stumpy forms. Fold changes of transcript levels for PAD1 (Tb927.7.5930), GPEET (Tb927.6.510), ESAG9 (Tb427.05.4620) and mitochondrial TAO (Tb927.10.7090) normalized to a control gene (Tb927.10.12970) determined by qPCR analysis. Total RNA was extracted from Antat 1.1 90:13 GFP-PAD1 parasites cultured in HMI-11 or HMI-11 supplemented with 68mM glycerol at several time points. This experiment was performed once without independent biological replicates.

Table 4.2 - Accumulation of stumpy forms during 30 hours, in the presence or absence of glycerol in the culture medium. Percentage of stumpy forms in Antat 1.1 90:13 GFP-PAD1 cultures supplemented with glycerol 68mM with an initial density of 10^5 parasites/mL assessed every 6 hours by flow cytometry.

		No glycerol				Glycerol			
Time points	0h	6h	12h	24h	30h	6h	12h	24h	30h
Stumpy forms (%)	4	3	2	2	2	5	9	20	33

After confirming that stumpy forms generated by exposure to glycerol *in vitro* were cell-cycle arrested and exhibited increased expression of some of the most important molecular markers of stumpy cells, we wanted to elucidate if these cells were viable and functionally capable of fulfilling their transmission potential. For this purpose, we tested their competence to fully differentiate to procyclic forms (PCFs), since only pre-adapted stumpy cells that have experienced the required metabolic and morphologic adaptations will be able to survive in the challenging environment of

the insect vector midgut. This developmental switch can be detected by shedding of the VSG surface coat and acquirement of a procyclin coat and can be reproduced *in vitro* by decreasing temperature, as well as pH and treating cells with cis-aconitate (an intermediate of the TCA cycle)[22,23].

To produce stumpy forms *in vitro,* parasite cultures were maintained for two days without passage in the presence of three different compounds: glycerol, pyruvate or CPT-cAMP. Pyruvate was tested in order to assess if it would also induce parasite differentiation similarly to glycerol, since it is the major end-product of glycolysis. Due to lack of a functional mitochondria and absence of lactate dehydrogenase[58] in BSFs to further metabolize pyruvate, this compound is rapidly excreted and accumulates substantially in the extracellular medium. Additionally, parasites were also induced with CPT-cAMP (a cell-permeable derivative of AMP) that would serve as a positive control, as this compound was previously described to be one of the effectors involved in the signaling pathway driving growth arrest *in vitro*[12]. Parasite growth and differentiation to stumpy forms were followed daily by counting cells in culture and quantifying GFP-PAD1 expressing cells by flow cytometry, respectively (**Figure 4.3A** and **Figure 4.3B**). After two days of incubation, 5 million parasites from each condition were transferred at high concentration ($1x10^6$ parasites/mL) to Differentiation Trypanosome Medium (DTM) containing 6mM cis-aconitate and incubated at 27˚C to trigger transformation to PCFs. The efficiency of this transition was assessed after 18 and 48 hours by quantification of procyclin on the parasites surface using an anti-procyclin antibody and measuring expression in parasites using flow cytometry (**Figure 4.3C** and **Figure 4.3D**).

It is clear that, in all conditions, supplementation of culture medium resulted in the decrease of parasite growth comparing to the control condition, which is probably due to early differentiation to stumpy forms. Glycerol was the compound capable of producing the highest percentage of GFP-PAD1 expressing cells in the first day of the experiment. After 24 hours of incubation, already 25% of the parasites induced with glycerol were committed to differentiation to stumpy forms, as opposed to only 9% and 11% induced with pyruvate and CPT-cAMP, respectively. The following day, the proportion of transmissible forms in cultures containing glycerol reached 78%, a percentage similar to that of CPT-cAMP supplemented cultures (86%). On the other hand, stumpy forms in cultures treated with pyruvate only accounted for nearly half of the parasite population (46%).

The functional capacity of these transmissible forms was then assessed through quantification of procyclin-expressing cells after transfer to conditions that partially reproduce the environment of the tsetse midgut. Regarding the first 18 hours, it is striking that approximatly 70% of the parasites present in cultures with added glycerol or the AMP derivative are procyclics,

while their counterparts in pyruvate-supplemented medium only account for approximately 30% of the population, which is comparable to standard HMI-11. As these percentages account for PCFs in the entire parasite population, the ratio of stumpy forms that converted into PCFs was determined to better draw conclusions. We can appreciate that glycerol contributes to formation of insect-transmissible forms that are prepared to differentiate to the insect stage as soon as they perceive the appropriate stimuli, as confirmed by the procyclic/stumpy ratio in culture of approximately 1 in the first timepoint. In contrast, only approximately half of the stumpy forms generated by incubation with pyruvate progressed to forms of the insect stage, while approximately 70% of CPT-AMP induced stumpy forms were competent for differentiating to PCFs.

Altogether we conclude that *in vitro* glycerol is the compound that induces the most efficient and rapid differentiation of slender forms to stumpy forms, which are fully functional insect-transmissible forms that readily transition to the insect stage.

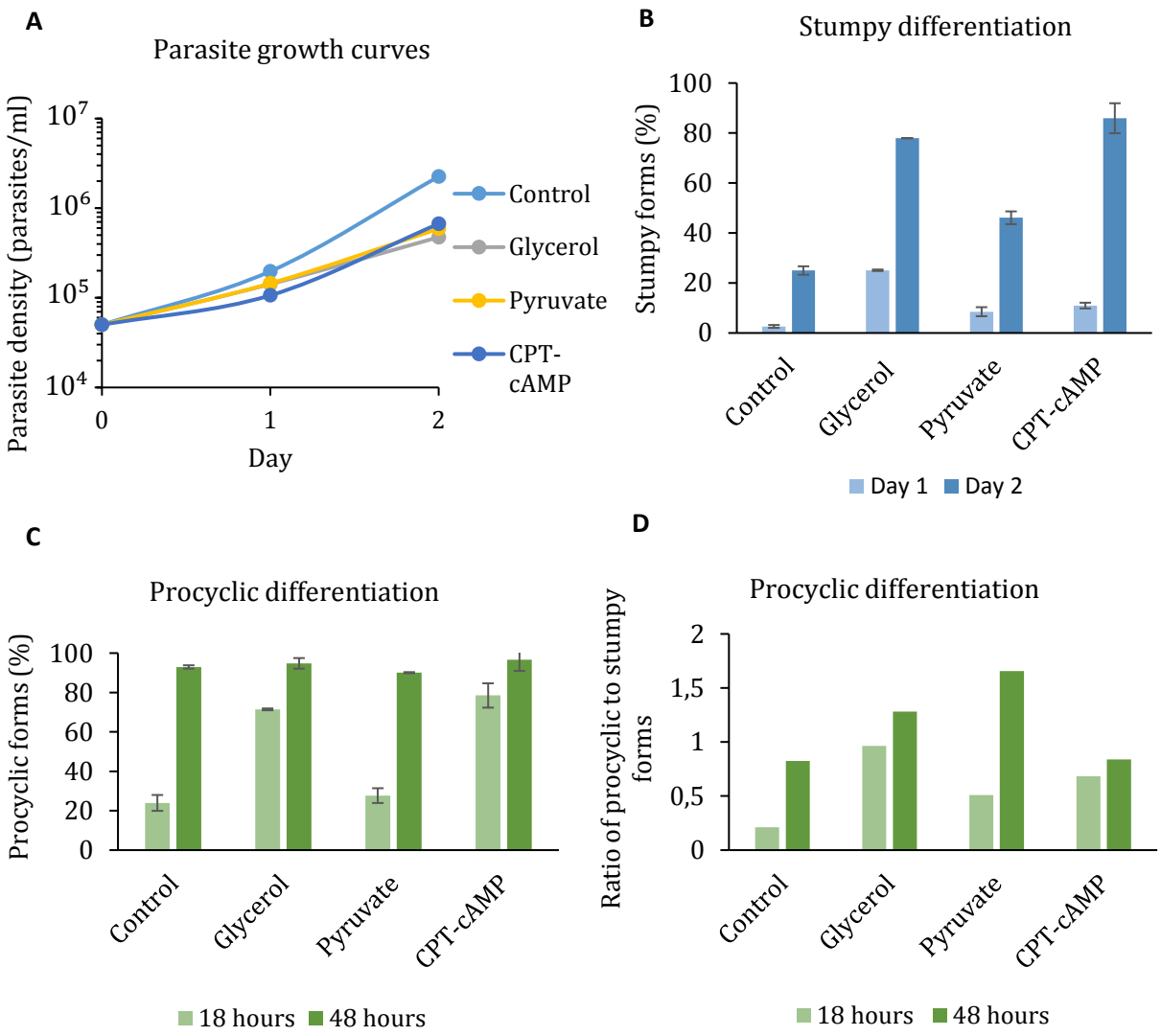

Figure 4.3- Glycerol induces stumpy forms competent for progression to the insect stage. AnTat 1.1 90:13 GFP-PAD1 cultures were initiated with a parasite density of $5x10^4$. (A) Parasite growth in cultures incubated with 68 mM glycerol, 6 mM pyruvate or 100 µM of an AMP analogue. (B) Percentage of Stumpy population reached after 1 and 2 days of culture in 68 mM glycerol, 6mM pyruvate and 100 µM cPT-cAMP. (C) Percentage of procyclin-expressing cells in culture determined by flow cytometry after 18 and 49 hours of parasite transfer to conditions that favor procyclic formation. (D) Ratio of the number of procyclic forms to the number of stumpy forms present in each condition. (A-C) Error bars represent standard deviation of the mean. Experiment was performed once with three to five biological replicates per condition.

4.1.3. Could glycerol be the stumpy induction factor?

So far, we have demonstrated that glycerol slows down parasite growth, promotes cell-cycle arrest of the parasite population in G0/G1 phase, increases the expression of stumpy molecular markers, enhances the number of GFP-PAD1 expressing cells, and ultimately triggers differentiation to fully competent stumpy forms. The next question is if glycerol produced and secreted by the parasite itself could also promote differentiation to stumpy forms *in vitro*, following the intrinsic characteristics of the Stumpy Induction Factor (SIF).

To understand whether glycerol is directly correlated with differentiation into stumpy forms, we investigated the effect of endogenous production of glycerol by the parasites associated to the proportion of insect-transmissible form. Towards that end, we maintained a culture of AnTat 1.1 90:13 GFP-PAD1 for two days in the absence of exogenous glycerol and without passing the cells. Parasite density, stumpy population and glycerol concentration present in the culture medium were monitored daily (**Figure 4.4)**. The parasite density increases exponentially during the first 24 hours of *in vitro* culture and reaches a *plateau* afterwards (**Figure 4.4A**). This plateau phase is accompanied by the increase of GFP-PAD1 expressing cells to 34% (**Figure 4.4B**). Therefore, as parasite density increases in culture and reaches a critical threshold, stumpy quiescent forms arise and limit cell numbers in culture, leading to a decrease in the growth rate of parasites. Interestingly, the concentration of glycerol released by the parasites in culture medium follows the same pattern as differentiation to stumpy forms. Indeed, the concentration of glycerol inside the culture medium is considered negligible during the first 24 hours and suddenly reaches 0,5mM after 52 hours of *in vitro* culture (**Figure 4.4C)**. Glycerol actually accumulates in culture medium as parasite density increases, exhibiting a positive correlation with stumpy formation *in vitro*, reinforcing that this parasite-derived metabolite has a behavior typical of a SIF.

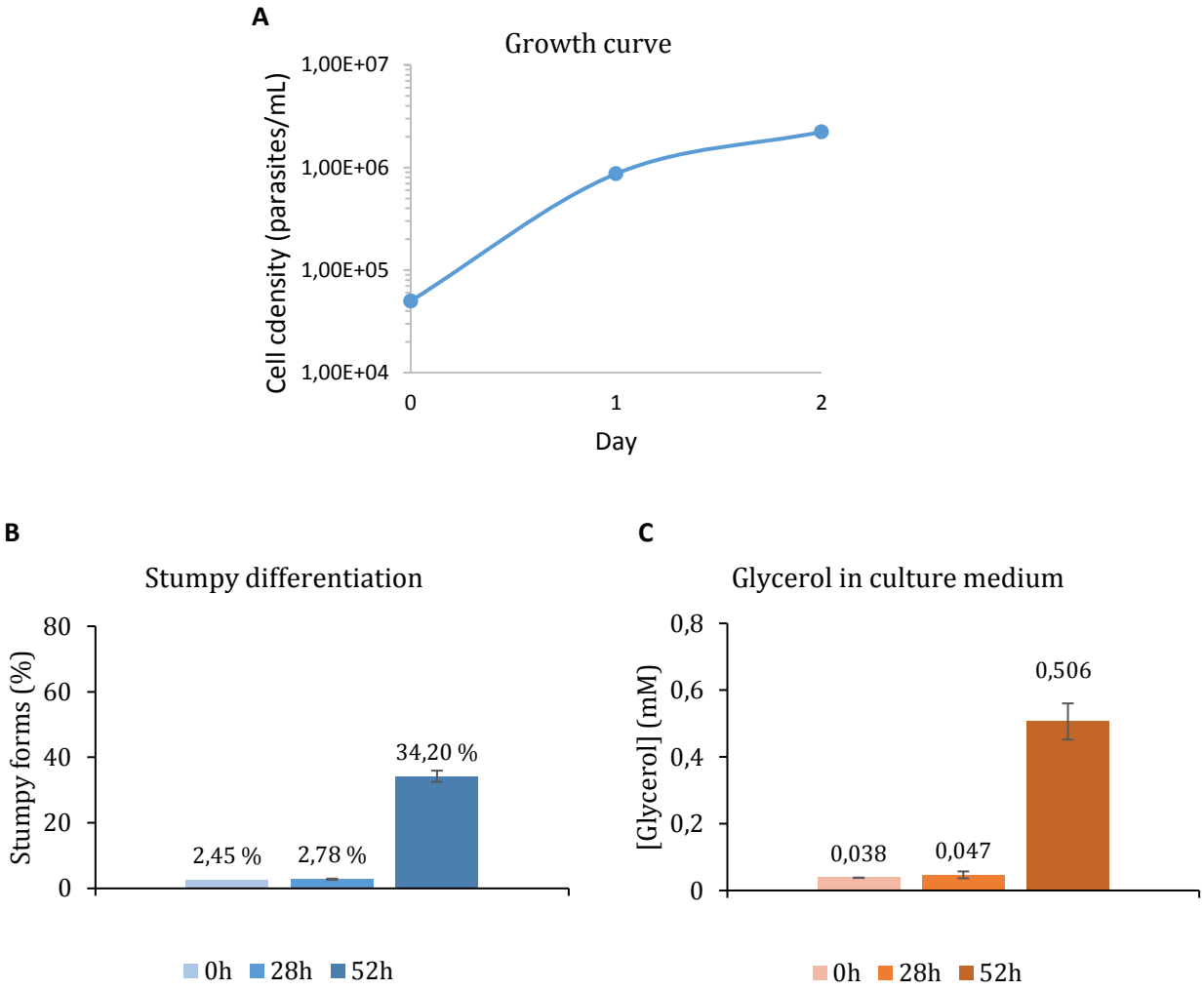

Figure 4.4 - Glycerol induces growth arrest and differentiation to stumpy forms upon accumulating in the culture medium. (A) Growth curve of AnTat 1.1 90:13 GFP-PAD1 parasites assessed daily by counting cells manually in a Neubauer chamber. (B) Differentiation to stumpy forms in AnTat 1.1 90:13 GFP-PAD1 culture throughout the time course assessed by quantification of GFP-PAD1 expressing cells through flow cytometry. (C) Glycerol quantified in culture medium collected throughout the time course. (B-C) Error bars represent standard deviation of the mean. This experiment was performed twice, each time with three biological replicates.

Following this lead, the potential of glycerol as the SIF was assessed by comparing the inducing effect of this compound with conditioned medium. It was previously described that transfer of culture medium from high density *T. brucei* cultures (termed conditioned medium) to cultures in which parasite density has not reached the threshold for differentiation is sufficient to greatly induce this process[12]. Cultures with a starting density that enables a negligible percentage of differentiation to stumpy forms were supplemented with either exogenous glycerol or with 50% conditioned medium prepared from monomorphic or pleomorphic strains after two days in culture. These cultures were maintained without passage for two days, during which parasite

density and percentage of stumpy forms were monitored daily using a Neubauer counting method and flow cytometry, respectively (**Figure 4.5**).

Conditioned medium from both pleomorphic and monomorphic cell lines had significant inhibitory effects on the growth of parasites fully capable of differentiating to stumpy forms, in comparison to the control condition (**Figure 4.5A**). Incubation of parasites with glycerol also resulted in appreciable delay in cell growth, although not as marked as in presence of conditioned medium, suggesting that maybe this is an additive effect of glycerol and other compound(s). After one day of incubation, cultures induced with glycerol exhibited 9% of stumpy population, while cultures incubated with conditioned medium showed 20% of differentiated forms, contrasting with only 3% of this population present in the control culture (**Figure 4.5C)**. Regarding the second day of the experiment, parasites induced by conditioned medium reached 94% of the population and parasites induced by glycerol reached 80% of the parasites, while differentiated forms induced by parasite density alone only accounted for 52% of the population. The last time point already showed over 90% of differentiated forms for both cultures supplemented with glycerol and conditioned medium, while non-supplemented cultures reached 80%. These observations support that glycerol has a similar activity to the SIF naturally present in conditioned medium, as evidenced by the fact that this metabolite promoted growth arrest and greatly enabled differentiation in pleomorphic parasites in a similar manner to conditioned medium.

Despite their inability to differentiate to transmissible forms, monomorphic parasites also experienced some degree of growth arrest between day 1 and day 2 of culture, induced by both conditioned medium and glycerol (**Figure 4.5B**). Monomorphic parasites demonstrated negligible progression of slender forms to stumpy forms despite their exposure to SIF (data not shown), as it would be expected since monomorphic strains are insensitive to the *quorum sensing* signal[12]. Interestingly, in parasites able to switch to transmissible forms, monomorphic conditioned medium triggered this process to the same extent as pleomorphic conditioned medium. Quantification of glycerol in conditioned medium from both pleomorphic and monomorphic strains indicated that monomorphic parasites are actually capable of secreting glycerol, displaying even greater concentrations of this metabolite extracellularly than their pleomorphic counterparts (**Figure 4.5D)**. Altogether, these evidences show that:

(1) – Glycerol could be the *quorum sensing* signal that is released to the culture medium by parasites upon reaching a critical density, thus triggering quiescence and parasite differentiation;

(2) – Although monomorphic parasites are only slightly responsive to this signal (SIF), they are able to produce glycerol and also release it to the surrounding medium.

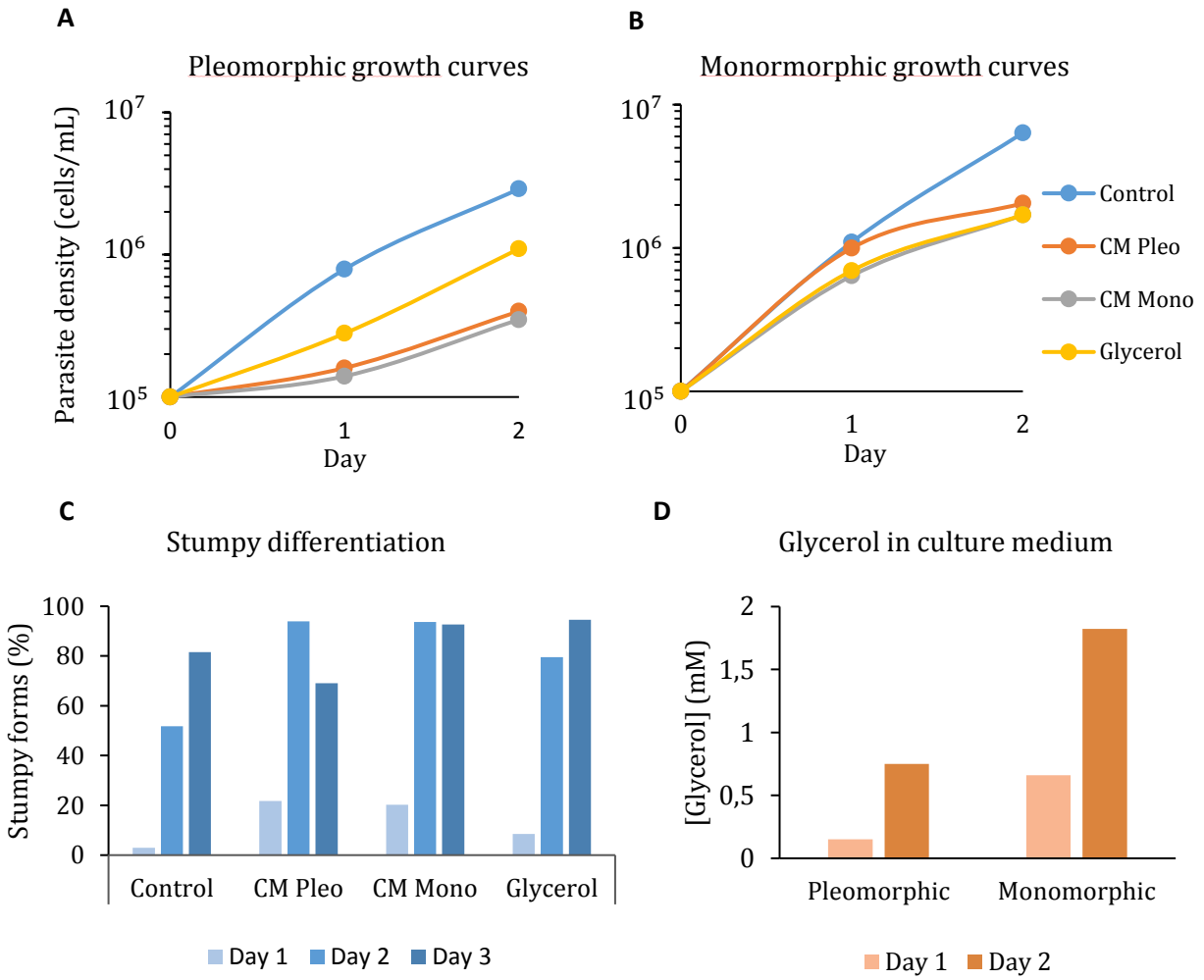

Figure 4.5 - Glycerol triggers growth arrest and differentiation to stumpy forms in *T. brucei* pleomorphic cultures, similarly to conditioned medium (CM). AnTat 1.1 90:13 GFP-PAD1 and PL1A GFP-PAD1 parasites were cultured in either 50% CM from a pleomorphic strain, 50% CM from a monomorphic strain or 68mM glycerol. Parasites were counted daily with a hemocytometer and differentiation was assessed through quantification of GFP-positive cells by flow cytometry. (A, B) Parasite density throughout the first two days of incubation in pleomorphic and monomorphic cultures, respectively. (C) Stumpy population percentage during 3 days of incubation. (D) Glycerol quantified in the culture medium collected during the three days of culture. This experiment was performed once without biological replicates.

4.1.4. Is glycerol sufficient to induce differentiation to stumpy forms?

To clarify if the inducing effect of glycerol is dependent on parasites reaching a critical density in culture, AnTat 1.1 90:13 GFP-PAD1 parasites were once again incubated with HMI-11 containing two different concentrations of glycerol (12 mM and 25 mM) and with different initial parasite densities in culture. Parasite concentration was determined daily using a Neubauer chamber and differentiation to stumpy forms was determined through quantification of the GFP signal using flow cytometry (**Figure 4.6**).

In the presence of 12 mM glycerol and at higher seeding densities (10^4 parasites/mL and 10^5 parasites/mL), parasites proliferate at a lower rate, as demonstrated by the growth curves heading towards the stationary phase in such cultures (**Figure 4.6A**). This behavior is more striking in culture medium containing 25 mM glycerol, since the parasites cultured with an initial density of 10^5 parasites/mL have already reached the stationary phase of their growth and are approaching a phase in which parasite numbers are declining (**Figure 4.6B**). As the initial seeding of parasites increases, the cumulative fold change of parasite density suffers a remarkable decrease, indicating that parasites are more responsive to glycerol when they reach higher parasite densities in culture (**Table 4.3**). The impact of glycerol in promoting transition of slender forms into stumpy forms is also greater as the initial parasite concentration increases, suggesting that the mechanism behind the effect of glycerol is somewhat dependent on parasite density (**Figure 4.7**).

On the other hand, increasing concentrations of glycerol have an earlier effect on differentiation to stumpy forms and growth arrest. The effect of glycerol on the differentiation to stumpy forms is dependent on the parasite density, suggesting that cells should be prepared to receive and respond to the quorum sensing signal, in this particular case, glycerol. Cultures initiated with 10^3 parasites/mL and 10^4 parasites/mL are already reaching a density *plateau* when incubated with HMI-11 containing 25mM glycerol as opposed to their counterparts incubated with 12 Mm glycerol.

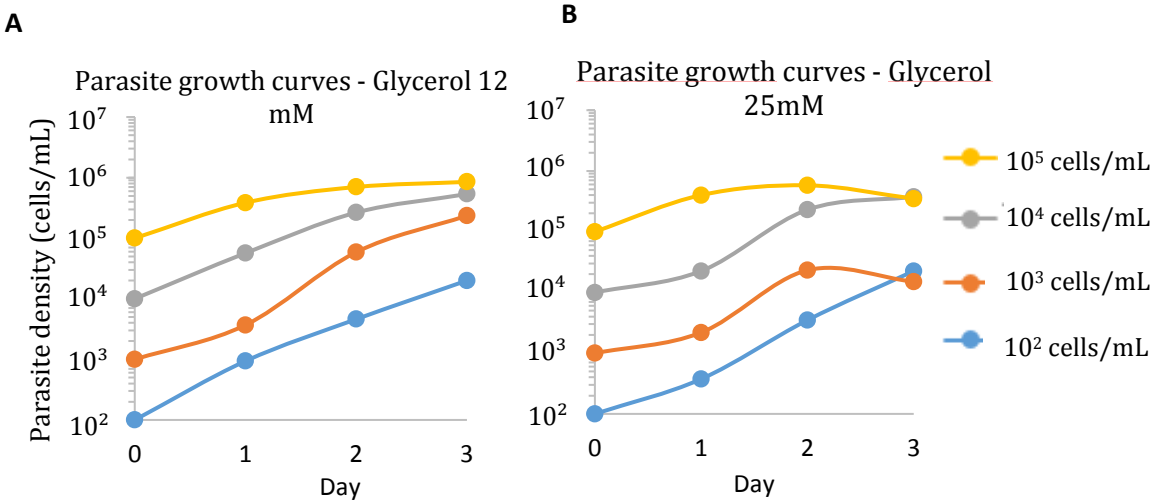

Figure 4.6 – Growth arrest promoted by glycerol is stronger with increasing initial density of parasites in culture. Parasites were counted daily using a hemocytometer. (A) Growth curves of AnTat 1.1 90:13 GFP-PAD1 parasites incubated with 12mM of glycerol. (B) Growth curves of AnTat 1.1 90:13 GFP-PAD1 parasites incubated with 25mM of glycerol. Parasites were counted daily using a Neubauer counting chamber. This experiment was performed one time with one biological replicate.

Table 4.3 - Effect of glycerol on growth arrest is stronger with increasing initial density of parasites in culture. Fold change of parasite density in AnTat 1.1 90:13 GFP-PAD1cultures incubated with 12mM glycerol or 25mM glycerol throughout the time course.

		Glycerol 12 mM				Glycerol 25mM			
Starting concentration (parasites/mL)		10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5
Fold change of parasite density compared to initial density	Day 1	9	4	6	4	4	2	2	4
	Day 2	46	59	27	7	35	23	23	6
	Day 3	200	235	54	9	225	15	38	4

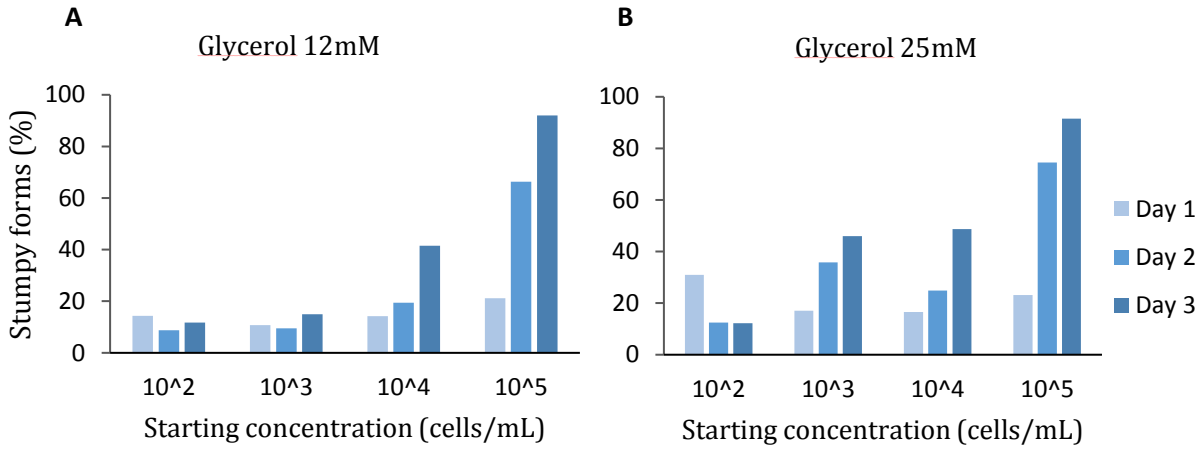

Figure 4.7 - Percentage of stumpy forms in cultures depends on glycerol concentration and parasite density. (A) Stumpy population in AnTat 1.1 90:13 GFP-PAD1 *T. brucei* cultures incubated with 12mM glycerol. (B) Stumpy population in AnTat 1.1 90:13 GFP-PAD1 *T. brucei* cultures incubated with 25mM glycerol. Experiment was performed one time with one biological replicate.

We deduce that glycerol is more effective at inducing differentiation to stumpy forms when parasites have reached a certain density threshold, meaning that the cells are not constitutively responsive to the *quorum sensing* signal. This might indicate that *T. brucei* needs to be prepared to perceive the signaling molecule triggering differentiation to stumpy forms.

4.1.5. Glycerol induces early upregulation of genes involved in glucose metabolism

Due to a highly repressed mitochondrion and absent respiratory chain, glycolysis is the sole metabolic pathway securing ATP production in BSFs, and it is consequently essential for parasite survival in the mammalian host. Interestingly, trypanosomes can utilize glycerol as an alternative carbon source to generate ATP under aerobic conditions when deprived of glucose[59]. Therefore, we assessed if supplementing glycerol to the culture medium as an additional carbon source, could affect the glycolytic flux in *T. brucei*, namely by modifying the expression of glycolytic enzymes. Additionally, the levels of glycosomal enzymes are also affected by autophagy of the glycosomes (resembling pexophagy) that accompanies differentiation of slender forms into stumpy forms[60]. This process is followed by generation of remodeled glycosomes accommodating a different enzymatic content, providing a mechanism for switching the metabolism in preparation for the insect stage of the life cycle.

For this experiment, pleomorphic parasites were either cultivated in the absence or with 68mM of glycerol in order to extract RNA at key time points and determine changes in transcript levels of certain glycolytic enzymes using a qPCR approach (**Figure 4.8**). Differentiation to stumpy forms was assessed during the time of culture by quantifying GFP-PAD1 expression using flow cytometry (**Table 4.4**). Hexokinase (HXK) and phosphofructokinase (PFK) are two of the first enzymes of glycolysis and both catalyze phosphorylation reactions inside the glycosome at the expense of ATP. Hexokinase is encoded in *T. brucei* by two genes with very similar sequences, TbHXK1 and TbHXK2[61]. Interestingly, all three genes exhibited approximately a 2-fold increase in transcript levels, after only 6 hours of incubating parasites with glycerol. After this time point, expression of TbHXK1, TbHXK2 and TbPFK declined in the presence of glycerol, correlating with an increase in stumpy forms that have partial activation of the mitochondrion and become less dependent on glycolysis. Despite the lack of feedback regulation of TbHXK1/2, their activity is appreciably decreased in acidic conditions which mimic fusion of lysosomes with glycosomes occurring during transition of slender forms to stumpy forms[62]. Curiously, the presence of glycerol-3-phosphate inhibits such pH-dependent inactivation of TbHXK1/2, a metabolite that is significantly accumulated in the glycosome through the reaction of glycerol kinase upon incubation with exogenous glycerol.

Transport of glucose in BSFs is mainly mediated by Trypanosome Hexose Transporter 1 (THT1) and this step exerts most of the control on the glycolytic flux[63]. Parasites exposed to glycerol displayed an increase of almost 3-fold in THT1 mRNA levels after 6 hours of incubation, followed by a slow decrease of the transcript levels to 2-fold of the constitutive transcript levels. However, THT1 expression did not decline to the levels determined at the first time point after 24

hours of culture with glycerol, which could be explained by a percentage of stumpy forms of only 20%, meaning that maybe regulation of this transcript occurs mainly during late differentiation to stumpy forms. Down-regulation of THT1 could serve as a pre-adaptation of stumpy cells for replacement of this glucose transporter by THT2 that takes place in PCFs.

Overall, these results are in accordance with decreased expression of 18 out of 43 glycolytic enzymes detected in stumpy forms relative to slender forms[65]. Throughout the time of incubation, parasites upregulated important genes involved in glucose metabolism as an early response to glycerol exposure. In the absence of glycerol, upregulation of the same genes encoding glycolytic enzymes occurs later in the time course, more specifically between 12 hours and 24 hours post-incubation, reinforcing that adding glycerol to the culture medium elicits differentiation of slender forms to stumpy forms even before reaching a critical density.

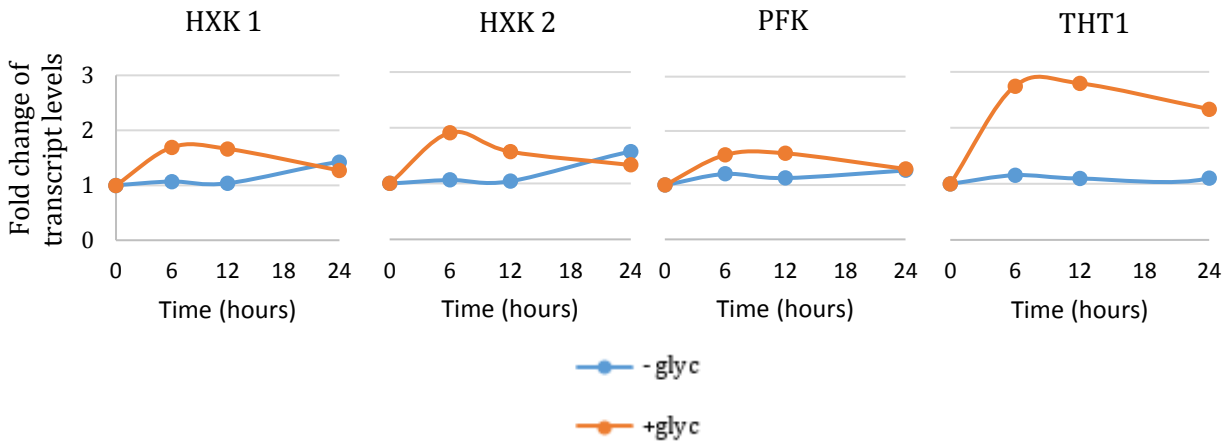

Figure 4.8 – Presence of glycerol *in vitro* leads to the upregulation of genes involved in glucose uptake and metabolism. Fold changes of transcript levels for HXK 1 (Tb927.10.2020), HXK 2 (Tb927.10.2010), PFK (Tb927.3.3270), and THT1 (Tb927.10.8440) normalized to a control transcript (Tb927.10.12970) determined by qPCR analysis. Total RNA was extracted from Antat 1.1 90:13 GFP-PAD1 parasites cultured in HMI-11 or HMI-11 supplemented with 68mM glycerol at several time points. Experiment was performed once without biological replicates.

Table 4.4 - Accumulation of stumpy forms during 30 hours, in the presence or absence of glycerol in the culture medium. Percentage of stumpy forms in Antat 1.1 90:13 GFP-PAD1 cultures supplemented with glycerol 68mM with an initial density of 10^5 parasites/mL assessed every 6 hours by flow cytometry.

		No glycerol				Glycerol (68 mM)			
Time points	0h	6h	12h	24h	30h	6h	12h	24h	30h
Stumpy forms (%)	4	3	2	2	2	5	9	20	33

4.1.6. Co-culture of *T. brucei* with adipocytes or fibroblasts induces differentiation to stumpy forms

We show that slender to stumpy transition is accompanied by a substantial production and release of glycerol by *T. brucei* parasites *in vitro*. Moreover, when added exogenously to *T. brucei* cultures, glycerol promotes growth impairment, G1 cell-cycle arrest of the parasite population and ultimately triggers differentiation to stumpy forms. Our laboratory has recently shown that adipose tissue is a major reservoir for *T. brucei* parasites during a mouse infection[8]. Adipose tissue, which is composed mostly of adipocytes, constitutes the main reservoir of triglycerides in the mammalian body. In states of fasting or exercise, adipocytes can provide energy to the body by mobilizing and catabolizing these triglycerides in a process termed lipolysis[66]. Triglycerides are converted into free fatty acids and glycerol that are released to the bloodstream via transport through the AQP7 channel[67]. Glycerol is then primarily metabolized in the liver by entering through AQP9 protein channels, followed by phosphorylation to gly-3-P by GK in order to be used for triglyceride synthesis and also gluconeogenesis[67]. Infection of *T. brucei* has been shown to increase the levels of triglycerides and other lipids present in the blood of infected animals[68], probably enhancing lipolysis and leading to subsequent weight loss and emaciation typically seen in affected animals[69].

Next, we investigated if exogenous glycerol produced by mouse adipocytes can promote differentiation to stumpy forms and if interaction between the two cell types can promote a greater release of glycerol. To accomplish this, we started a co-culture of Antat 1.1 90:13 GFP-PAD1 parasites with either 3T3-L1 fibroblasts or its differentiated adipocyte version (using differentiated cocktail). During the time of co-cultures, glycerol concentration in the culture medium and differentiation to stumpy forms of *T. brucei* parasites were quantified daily (**Figure 4.9**).

As expected, glycerol is only detected in the culture medium of differentiated adipocytes and the concentration of this metabolite increased during the time of culture from 0,33 mM at day 1 to 0,98 mM at day 3 (**Figure 4.9A**). Comparing to adipocytes, *T. bruc*ei parasites produce and release more glycerol into the culture medium, secreting 2 mM of glycerol after 3 days. Interestingly, when establishing cell-to-cell interactions with 3T3-L1 fibroblasts, *T. brucei* cultures exhibit higher levels of glycerol comparing to the single parasite culture. On the other hand, adipocyte and *T. brucei* co-cultures only displayed a higher glycerol concentration than single parasite culture in the first day of incubation. However, glycerol concentration in co-cultures of adipocytes and *T. brucei* is higher that mono-cultures of adipocytes.

Co-culture systems show a higher rate of parasite differentiation comparing to single parasite cultures. Interestingly, the rate of differentiation to stumpy forms correlated strongly with

glycerol concentration in the culture medium, in which higher glycerol levels showed higher proportion of insect-transmissible parasites in culture (**Figure 4.9B)**. Altogether, these results strongly link stumpy formation *in vitro* with glycerol concentration present inside the culture medium.

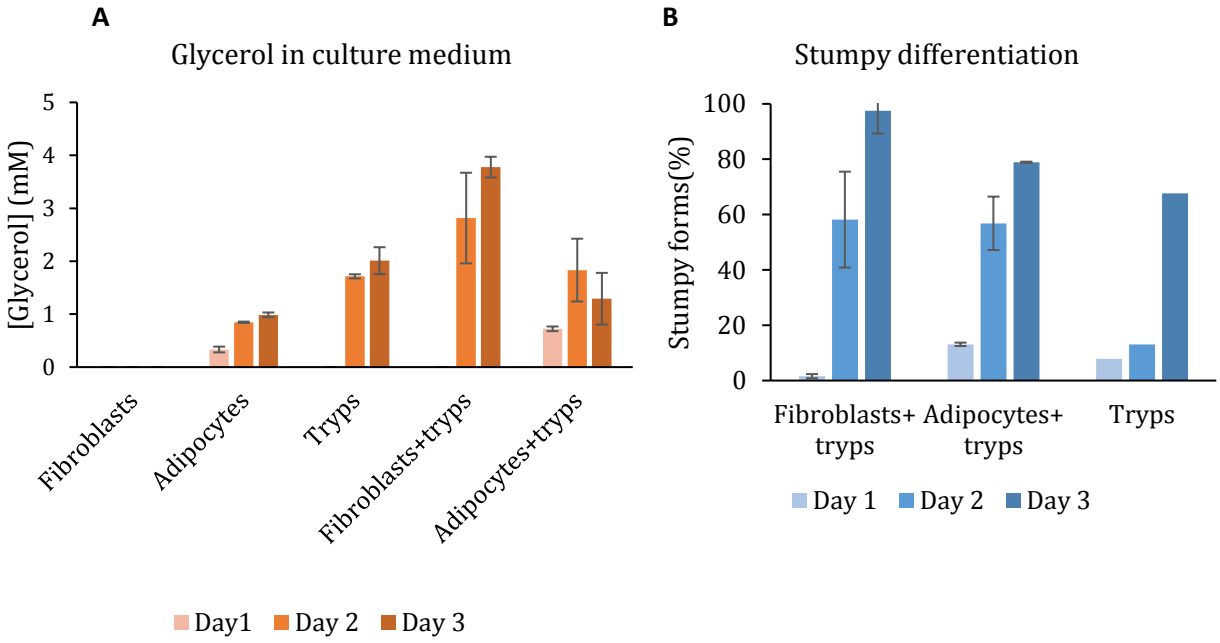

Figure 4.9 – Co-culture of *T. brucei* with fibroblasts or adipocytes induces differentiation to stumpy forms. (A) Glycerol quantification in culture medium from cultures of fibroblasts, adipocytes and T. brucei and also co-cultures of T. brucei with adipocytes and *T. brucei* with fibroblasts. (B) Quantification of stumpy population in Antat 1.1 90:13 GFP-PAD1 culture and co-cultures of Antat 1.1 90:13 GFP-PAD1 with either adipocytes or fibroblasts. (A-B) Error bars represent standard deviation of the mean value. Experiment was performed once with one or three biological replicates).

4.2. Generation of parasites impaired for glycerol production or transport

So far, we have concluded that glycerol assumes an important role in promoting the developmental transition of slender forms to stumpy forms. We have demonstrated that the concentration of glycerol secreted by parasites in the culture medium correlates positively with the extent of differentiation to stumpy forms. The insect-transmissible forms produced by incubation with this glycolytic end-product display some of the most recognizable hallmarks of stumpy forms: cell-cycle arrest in G1 phase, upregulation of specific molecular markers of stumpy forms, and capacity to rapidly differentiate to PCFs. To better understand the molecular mechanism through which glycerol induces differentiation to stumpy forms *in vitro*, we applied loss-of-function approaches to create mutant parasites impaired in glycerol metabolism or transport (**Figure 4.10**).

Ablation of glycerol production inside the glycosome was achieved through targeting of the glycerol kinase mRNA by RNA interference (RNAi). This enzyme catalyses the conversion of glycerol to glycerol-3-phosphate, but can also perform the reverse reaction in *T. brucei*, proving to be useful to parasites when in anaerobic conditions by providing ATP through conversion of Gly-3-P to glycerol, the latter being rapidly excreted by the parasite[38]. However, this reaction is thermodinamically unfavorable under aerobic conditions, occuring only when a glycerol-3-phosphate pool accumulates inside the glycosome. We expect that depletion of this enzyme should lead to negligible levels of glycerol both intra and extracellularly, impacting the *quorum sensing* signalling performed by glycerol that occurs upon reaching a threshold for parasite density.

Glycerol release by parasites and accumulation in conditioned medium can also be abrogated through inhibition of protein mediated transport of this glycolytic metabolite to the extracellular space. In *T. brucei,* three AQP channels account as the major route for influx and efflux of glycerol and are non-essential for these parasites[45,47,]. Therefore, all three genes enconding for these membrane transporters were deleted and replaced by drug resistance genes. Parasite differentiation, parasite growth and also the levels of both end-products of glycolysis (glycerol and pyruvate) were assessed in the two mutant cell lines that are impaired for either glycerol production or transport.

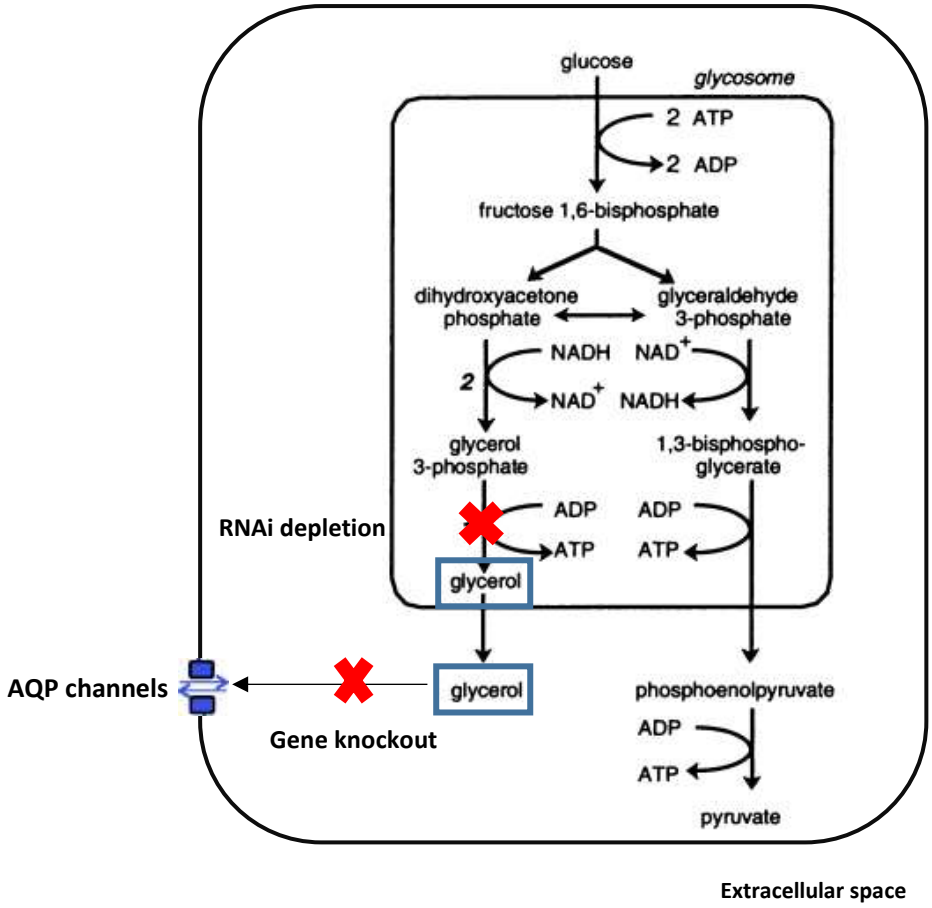

Figure 4.10 - Schematic representation of the glycolytic pathway in bloodstream forms and strategy to inhibit glycerol production and glycerol release in *T. brucei*. Inhibition of glycerol metabolism was mediated by depletion of glycerol kinase (GK) via RNAi depletion, while abolition of the facilitated transport of glycerol across the plasma membrane was performed by deleting all three genes encoding for aquaglyceroporins in *T. brucei.* Adapted from 38.

4.2.1. Generation and validation of AQPs *T. brucei* null mutants

Besides differentiation to stumpy forms being triggered by extensive production and secretion of glycerol by *T. brucei* during proliferation, addition of exogenous glycerol to the culture medium also promotes the developmental switch towards stumpy forms. Next, we asked whether inhibition of glycerol transport across the parasite plasma membrane would abolish parasite differentiation to stumpy forms *in vitro*.

In *T. brucei*, the influx and efflux of glycerol is mainly mediated by AQPs channels which are encoded by three different genes (TbAQP1, TbAQP2, and TbAQP3)[45]. Our goal was to generate a triple knock-out cell line for all three AQP genes in an Antat 1.1 GFP-PAD1 cell line and to study its phenotype for differentiation to stumpy forms. Firstly, we started by deleting TbAQP2 and TbAQP3 genes, which are adjacent genes located on chromosome 10. As *T. brucei* has a diploid genome, replacement of TbAQP2-3 locus by drug resistance cassettes in both alleles was performed by two sequential DNA transfections. (**Figure 4.11A**). Afterwards, we selected three

independent clones and confirmed double knockout of both genes by amplification of the genomic DNA using specific primers (**Table 3.2**) and performing electrophoresis of the PCR products (**Figure 4.11B**).

In order to confirm the genotyping of the three AQP2-3 knockout clones, total RNA was extracted and the AQPs transcript levels were quantified by qPCR using gene-specific primers (**Table 3.2** and **Figure 4.11C**). Clone 2 and clone 3 showed the greatest decrease of the levels of AQP2 and AQP3 transcripts (of approximately a hundred-fold comparing to the levels exhibited by the parasites from the parental cell line), indicating that both genes were successfully deleted. In contrast, clone 1 exhibited only a reduction of less than ten times the AQP2-3 mRNA present parental cell line that could be explained by the presence of contaminations in the sample or unspecific hybridization of the primers. Moreover, as expected, none of the three knockout clones showed significant changes in AQP1 transcript levels.

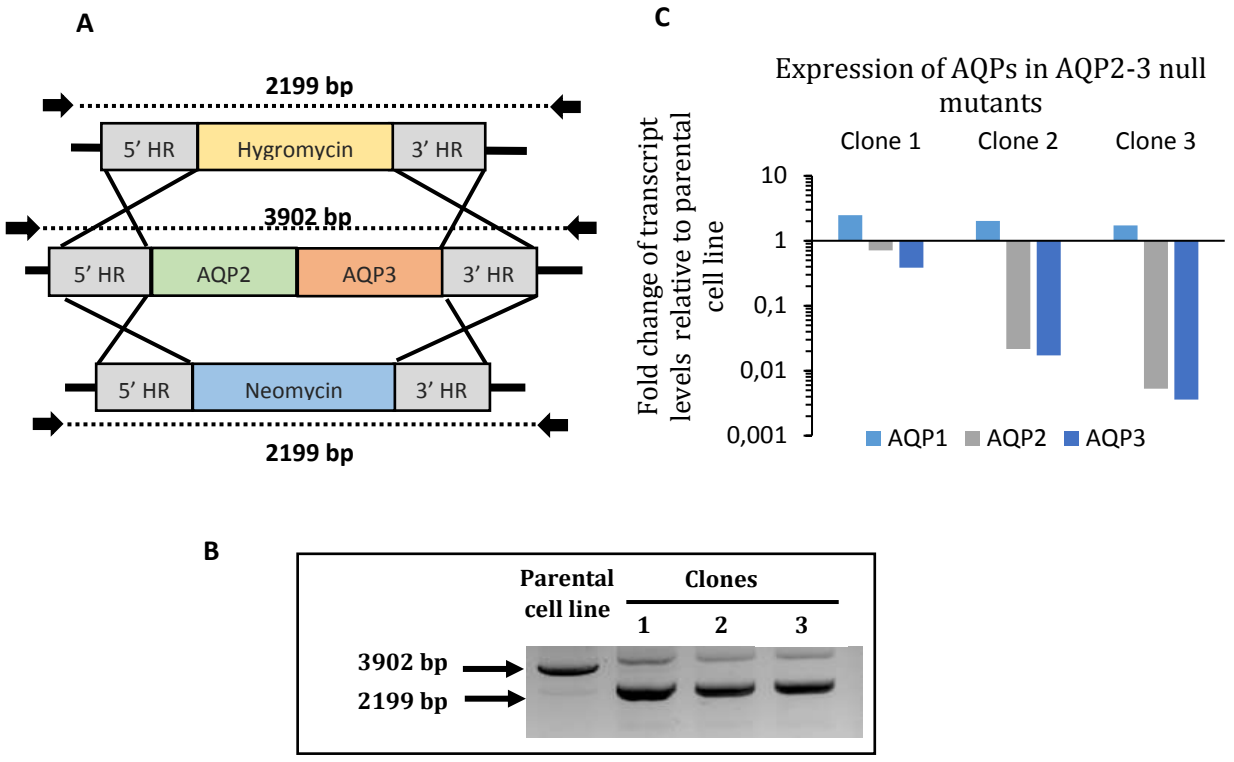

Figure 4.11 – Generation and validation of AQP2-3 double-knockout mutants. (A) Diagram of the strategy used for deleting both copies of AQP2 and AQP3 in *T. brucei* genome by replacement of the gene sequences with drug resistance cassettes. Amplicon sizes for the AQP2-3 locus before and after replacing these genes with the drug resistance cassettes are also represented (B) Agarose gel of the amplification products of genomic DNA extracted from AnTat1.1 GFP-PAD1 (parental cell line) parasites and three AQP2-3 double knockout mutants. Observation of a smaller DNA band at 2199 base pairs for the AQP2-3 null mutants indicates replacement of the targeted locus by a smaller fragment. (C) qPCR analysis of AQP1, AQP2 and AQP3 expression in three AQP2-3 double knockout mutants relative to wild-type cells. Values above 1 indicate overexpression of genes comparing to the parental cell line parasites and values below 1 represent lower expression of genes relative to the parental cell line parasites

Therefore, we selected clone 3, which displayed the greatest level of depletion of AQP2/3 transcripts to pursue and generate the triple AQP knockout mutant cell-line. We followed the same approach as before by knocking out and replacing the two copies of the AQP1 gene with two other drug resistance genes (**Figure 4.12A).** PCR genotyping confirmed the deletion of the AQP1 gene (band at approximately 2555bp in parental cell line) and its replacement by the two drug resistance genes (puromycin located at 1850bp and phleomycin at approximately 2084bp) (**Figure 4.12B).** Interestingly, clone 3 displayed a different DNA pattern from the other clones. To confirm the genotyping of our AQP triple knock-out mutant cell-lines, we performed qPCR analysis using gene specific primers for each APQ transcripts (**Table 3.2** and **Figure 4.12C)**. All three AQP triple knock-out clones showed a depletion of each AQPs transcript levels of at least a thousand times, confirming that AQP genes are no longer expressed in these mutants.

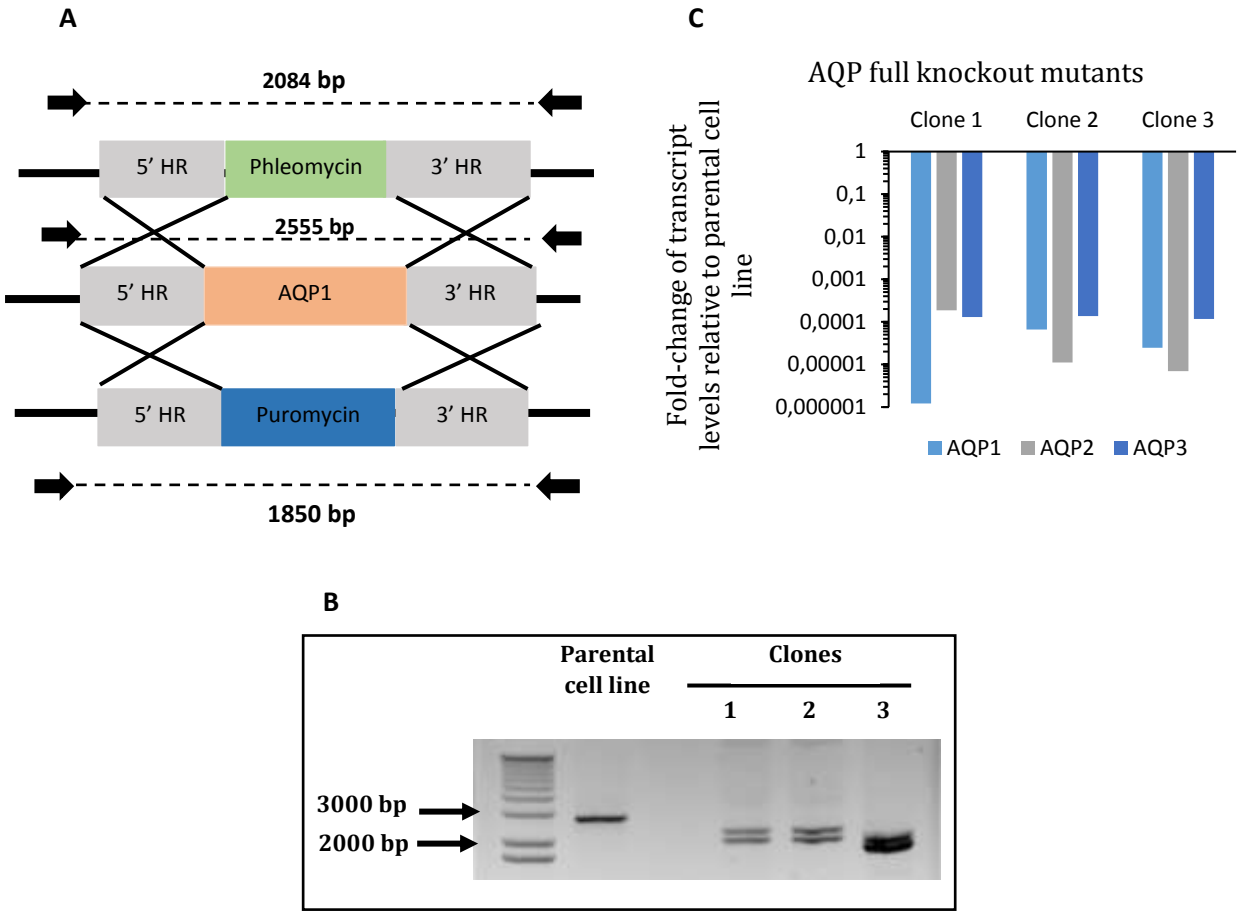

Figure 4.12 – Generation and validation of triple null AQP mutants. (A) – Diagram of the strategy used for deleting both copies of AQP1 in *T. brucei* genome by replacement of the gene sequences with drug resistance cassettes. Amplicon sizes for the AQP1 locus before and after replacing this gene with the drug resistance cassettes are also represented. (B) – Agarose gel of genomic DNA extracted from EATRO 1125 AnTat1.1 GFP-PAD1 wild-type parasites and three AQP1-3 double allele knockout mutants. (C) – qPCR analysis of AQP1, AQP2 and AQP3 expression in three AQP1-3 double knockout mutants relative to wild-type cells. Values above 1 indicate overexpression of genes comparing to the parental cell line parasites and values below 1 represent lower expression of genes relative to the parental cell line parasites.

4.2.2. Loss of AQPs in *T. brucei* has no impact on differentiation to stumpy forms

Next, we investigated the phenotype associated to AQPs knockout in *T. brucei*, by evaluating parasite growth, glycerol transport and the capacity of these mutant parasites to differentiate into stumpy forms. It was previously reported that these membrane channels are dispensable for parasite survival, but their deletion hinders glycerol uptake by the cells and also efflux to the extracellular space[47]. We expect AQPs null mutant parasites to be defective in differentiation to stumpy forms due to their inability of excreting and importing glycerol, a metabolite that we showed to promote slender to stumpy transition *in vitro*.

AQP1-3 null parasites were cultivated for three days without passage at a starting density of $5x10^4$ parasites/mL. Differentiation from slender forms to stumpy forms was determined daily by quantification of GFP-PAD1 expressing cells through flow cytometry and parasite densities were assessed using a Neubauer chamber. Additionally, the release of glycerol and pyruvate by the parasites was quantified throughout the time course using commercially available kits. (**Figure 4.13**). First, we confirmed that glycerol transport is impaired in the AQP KO mutant cell line (**Figure 4.13C**). Indeed, while glycerol concentration increases in the culture medium overtime in the control condition, levels of this metabolite secreted to the culture medium by AQP KO parasites are more are less maintained (below 0.5mM). Moreover, depletion of AQPs does not affect either the production or transport of pyruvate in the mutant parasites (**Figure 4.13D**). Surprisingly, when we measured parasite density and the percentage of GFP-PAD1 expressing cells, we did not detect any differences between the parental cell line and the AQP null mutant parasites (**Figure 4.13A** and **Figure 4.13B)**. Overall, these results show that AQP channels impair glycerol efflux, but are not essential for differentiation to stumpy forms in *T. brucei*. Nevertheless, we cannot exclude that low contents of glycerol released by AQP KO mutants could be sufficient to promote differentiation to stumpy forms. On the other hand, as a result of transport defect, glycerol could accumulate inside the parasite and still trigger differentiation to stumpy forms.

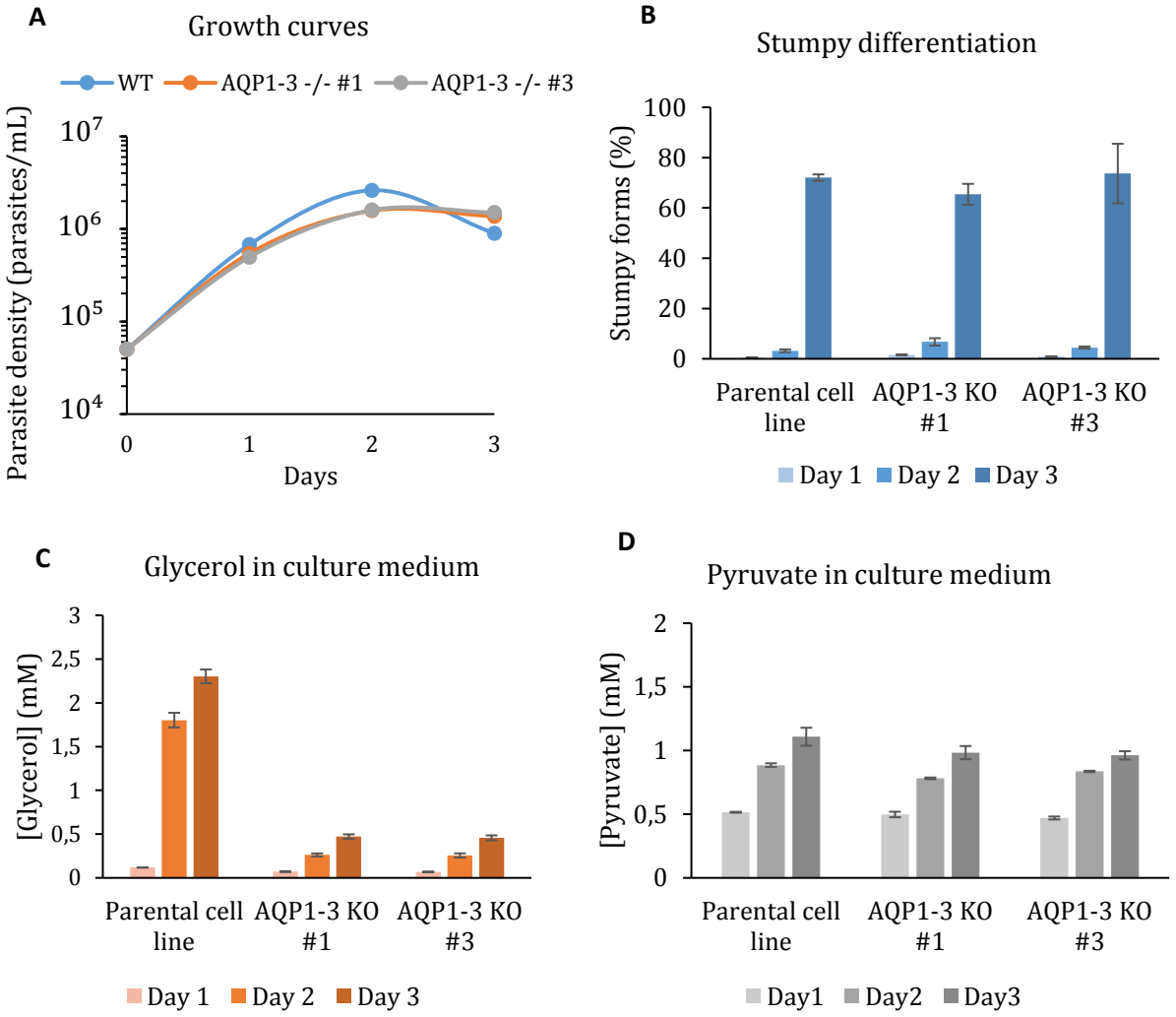

Figure 4.13 – Loss of glycerol transporters (AQPs) has no impact on parasite differentiation. (A) Parasite growth of AnTat 1.1 GFP-PAD1 (parental cell line) and AQP1-3 null mutants for 3 days of culture with a starting density of 5x10⁴ parasites/mL. (B) Differentiation to stumpy forms of AnTat 1.1 GFP-PAD1 and AQP full knockout parasites throughout the time course. (C) Glycerol quantified daily in culture medium. (D) Pyruvate quantified daily in culture medium. (B – D) Error bars represent standard deviation from mean. Experiment was performed two times with three biological replicates.

4.2.3. Generation and validation of glycerol kinase RNAi mutants

Although impairment in glycerol transport did not alter the ability of *T. brucei* parasites to differentiate into stumpy forms, we postulated that a defect in glycerol production by the parasite would affect differentiation to stumpy forms. Therefore, we decided to target the single metabolic enzyme that can anabolize and catabolize glycerol in *T. brucei*, glycerol kinase (GK). Since we did not know if glycerol kinase (Tb927.9.12550) was essential to parasites, we decided to deplete this enzyme at the mRNA level using an RNAi strategy (knockdown).

We transfected AnTat1.1 90:13 GFP-PAD1 cell line with an inverted-repeat construct targeting the GK gene[70]. This construct produces a long stem-loop RNA which activates and guides the RNAi machinery of *T. brucei* to specifically degrade GK mRNAs, resulting in glycerol kinase deficient parasites. We selected two independent clones of glycerol kinase RNAi mutant parasites and induced GK depletion upon treatment with tetracycline for two days *in vitro*. RNA and proteins were extracted and analyzed specifically for GK depletion using qPCR and Western blotting strategies, respectively (**Figure 4.14**). However, this analysis was only performed for clone 1 due to degradation of the RNA samples prepared from the other clones. Protein levels of glycerol kinase were also estimated in parasite lysates from clones 1 and 2 by Western blotting. qPCR and Western blotting analysis showed that only clone 1 of GK RNAi mutant cell line has strong depletion for both GK transcript and protein level (**Figure 4.14A** and **Figure 4.14B**). In contrast, clone 2 shows no depletion of GK at the protein level (**Figure 4.14B**). However, the RNAi construct in clone 1 is not tightly regulated, as the transcript levels of non-induced parasites are not similar to those of the parental cell line. In conclusion, we successfully generated GK RNAi mutant parasites that promote GK depletion at the mRNA and protein level, upon tetracycline activation. Therefore, we select clone 1 of GK RNAi mutant parasite to study the phenotype in differentiation to stumpy forms associated to GK depletion.

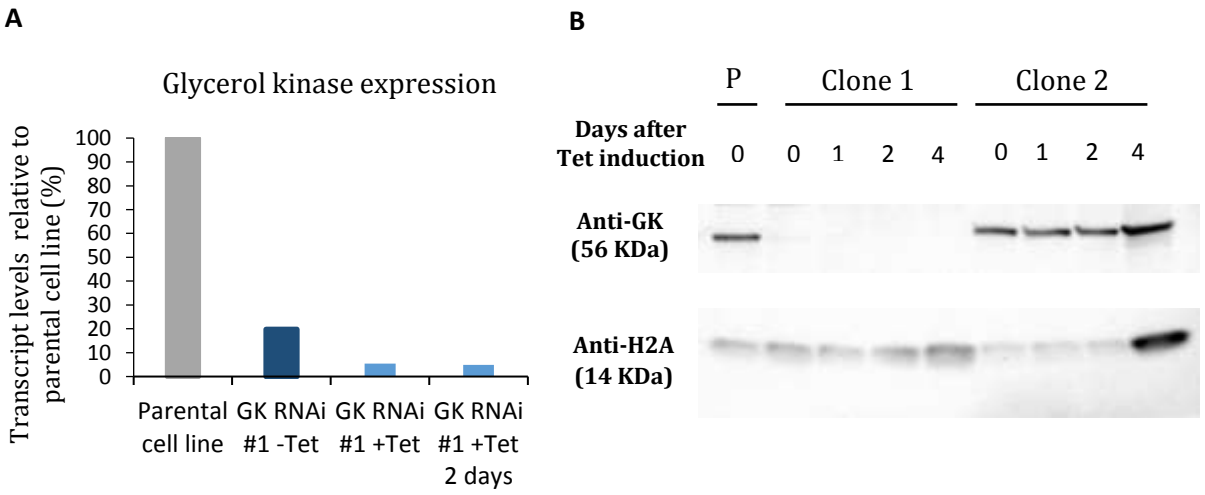

Figure 4.14 – Relative quantification of glycerol kinase expression in RNAi clones. (A) qPCR analysis of glycerol kinase (Tb927.9.12550) transcript levels relative to those of the parental cell line. (B) Western blot of total protein extracts from the parental cell line and the GK RNAi clones performed for detection and quantification of GK protein. P – Parental cell line

4.2.4. Depletion of glycerol kinase promotes differentiation to stumpy forms

Next, we wanted to know if impairment of glycerol production would alter the differentiation to stumpy forms process in *T. brucei*. To that end, GK RNAi mutant parasites were cultivated during 3 days in the presence or absence of tetracycline to induce or not the RNAi machinery. Clone 2 of the GK RNAi cell line was used in these experiments as a control of non-depleted GK parasites, since it did not exhibit depletion of this enzyme. Glycerol and pyruvate concentrations, parasite growth and differentiation to stumpy forms were quantified daily using respectively commercial kit, Neubauer chamber and flow cytometry (**Figure 4.15**).

First, we confirmed that depletion of glycerol kinase abolishes glycerol production in *T. brucei* parasites. Indeed, while glycerol accumulates in the culture medium of the parental cell-line and GK RNAi clone 2 parasites (reaching 1,4mM at day 3), glycerol concentration remains at low levels (below 0,11mM) when cultures of GK RNAi clone 1 parasites are induced with tetracycline (**Figure 4.15C**). Surprisingly, abolishment of glycerol production is accompanied with a reduction of parasite growth (**Figure 4.15A**) and premature differentiation to stumpy forms (**Figure 4.15B**). Indeed, tetracycline induced GK RNAi clone 1 parasite shows a percentage of 50% of stumpy cells at day 2 of culture, while parental cell-line and GK RNAi clone 2 parasites have less than 20% of stumpy forms (Figure 4.15B). Hence, abolishment of glycerol production leads to a premature differentiation to stumpy forms in *T. brucei*. Moreover, this premature differentiation "mediated" by the depletion of GK, seems independent of pyruvate production and secretion. Indeed, no changes in pyruvate concentration could be observed between the parental cell line and the GK RNAI clone 1 parasites (**Figure 4.15D**).

Given that adding exogenous glycerol to a culture triggers premature differentiation to stumpy forms, we were not expecting that a mutant that produces less glycerol would lead to a more efficient differentiation. However, glycerol kinase enzyme functions bi-directionally in *T. brucei* producing glycerol-3-phosphate from glycerol and also catalyzing the reverse reaction[38]. Therefore, it is possible that depletion of glycerol kinase enzyme could result in the accumulation of glycerol-3-phosphate (G3P) inside *T. brucei* parasites. Future studies are necessary to decipher the role of G-3-P in triggering the generation of stumpy forms in *T. brucei*.

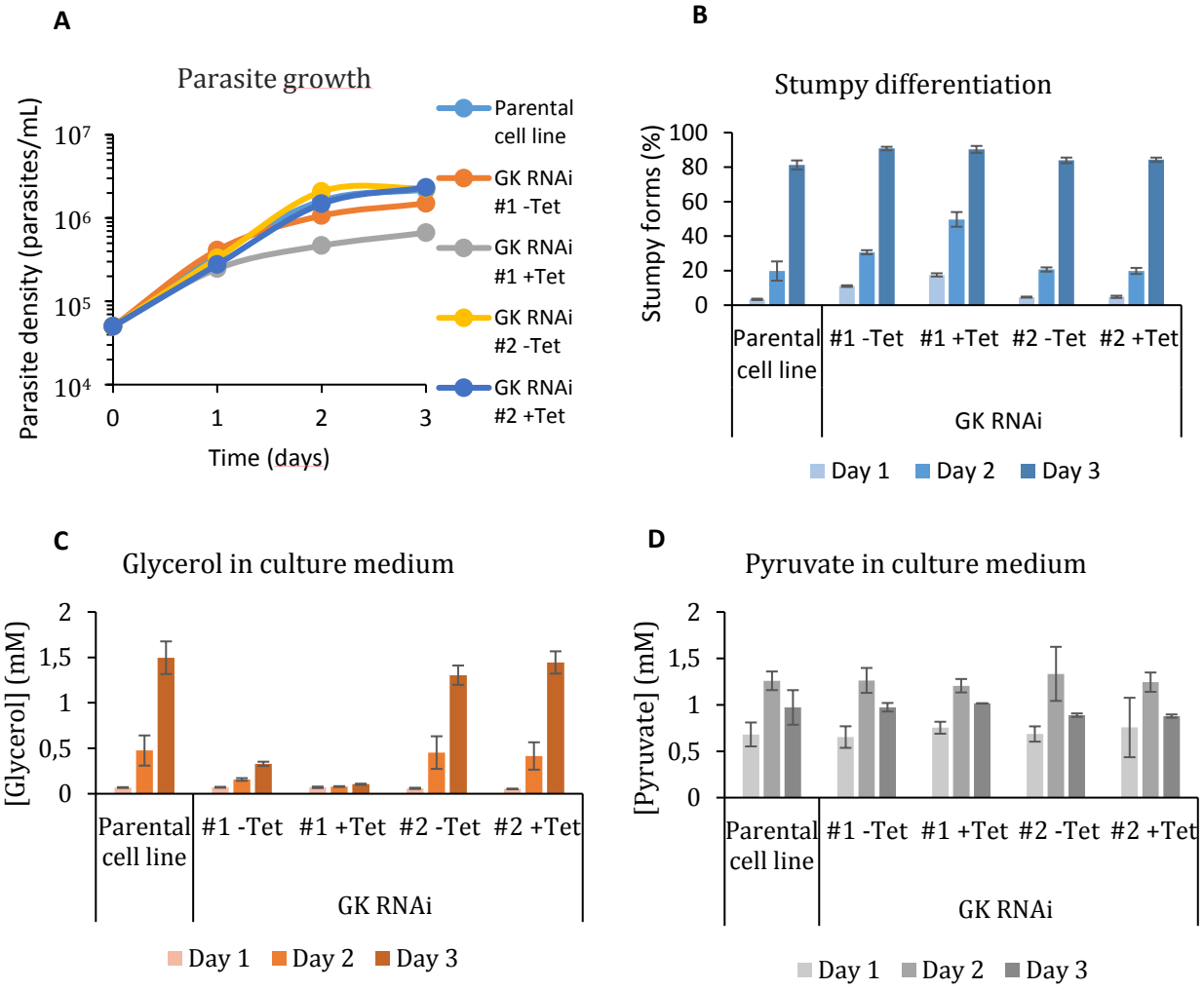

Figure 4.15 – Glycerol kinase RNAi mutants differentiate to stumpy forms earlier than WT parasites. (A) – Parasite growth during 3 days of culture with a starting density of $5x10^4$ parasites/mL. (B) – Differentiation to stumpy forms of glycerol kinase RNAi mutants induced and non-induced with tetracyclin throughout the time course. (C) – Glycerol quantified daily in culture medium daily and normalized to cell number. (D) – Pyruvate quantified daily in culture medium daily and normalized to cell number. (B-D) Error bars represent standard deviation from mean. Experiment was performed once with three biological replicates).

4.2.5. Glycerol metabolism is important for sustaining *T. brucei* infection in mice

Glycerol metabolism plays a crucial role in differentiation to stumpy forms of *T. brucei* parasites *in vitro*. Indeed, we have shown that either the addition of exogenous glycerol or the depletion of *T. brucei* glycerol kinase lead to a premature progression of parasites to insect-transmissible forms in culture. Interestingly, these phenotypes could in both cases be due to an accumulation of glycerol-3-phosphate metabolite inside the parasite. Next, we investigated if glycerol metabolism would influence the dynamics of *T. brucei* infection *in vivo* (**Figure 4.16**). We set up two groups of mice infected either with the parental cell-line (AnTat 1.1 90:13 GFP-PAD1) or GK RNAI clone 1 mutant parasites. Moreover, the group of mice infected with GK RNAi parasites were divided into two cages. One cage had normal water supply and the other one had doxycycline supplemented in the water to promote expression of the RNAi construct in parasites. We would expect for this mutant cell line to render a lower parasitemia due to higher proportions of quiescent forms compared to the parental cell line, as demonstrated *in vitro*.

The parental A90:13 GFPPAD1 cell line shows a typical pattern of *T. brucei* infection in mice with successive waves of parasitemia until the animal death around day 35 (**Figure 4.16A** and **Figure 4.16B**). In contrast, mice infection with doxycycline induced GK RNAi mutant parasites showed a completely different pattern with rarely detectable parasitemia and 100% mice survival. Interestingly, mice infected with induced GK RNAi parasites show only one first small peak of parasitemia before parasites disappear completely from the mouse blood. Strikingly, parasitemia was already undetectable at day 5 post-infection, when usually the number of circulating parasites reaches a peak and differentiation to stumpy forms by a density-sensing mechanism takes place. However, we have shown that GK RNAi mutant cells differentiate earlier to tsetse-infective forms comparing to the parental cell line *in vitro*. Knowing that stumpy forms are non-proliferative cells, a premature differentiation to stumpy forms *in vivo* would lead to a smaller and abortive parasitemia as already described before[15].

Notably, non-induced GK RNAi parasites show an intermediate phenotype, both in terms of parasitemia and mice survival. This is probably due to the lack of tight regulation of the RNAi construct, leading to leaky expression of the RNAi construct and those low levels of depletion. Overall, infection carried with parasites depleted of glycerol kinase resulted in lower parasitemia in mice and also increased host survival. These mutants delayed appreciably lethality comparing to the parental cell line parasites, especially when induced with doxycycline. From day 35 post-infection onwards, survival of mice infected with wild-type parasites declined rapidly, while loss of mice infected with glycerol kinase depleted parasites was only observed 52 days post-infection (25%). Most importantly, no mice died from infection with induced GK RNAi parasites. Altogether,

these evidences show that glycerol metabolism is also crucial for the dynamic of *T. brucei* infection in mice, probably due to premature differentiation to stumpy forms.

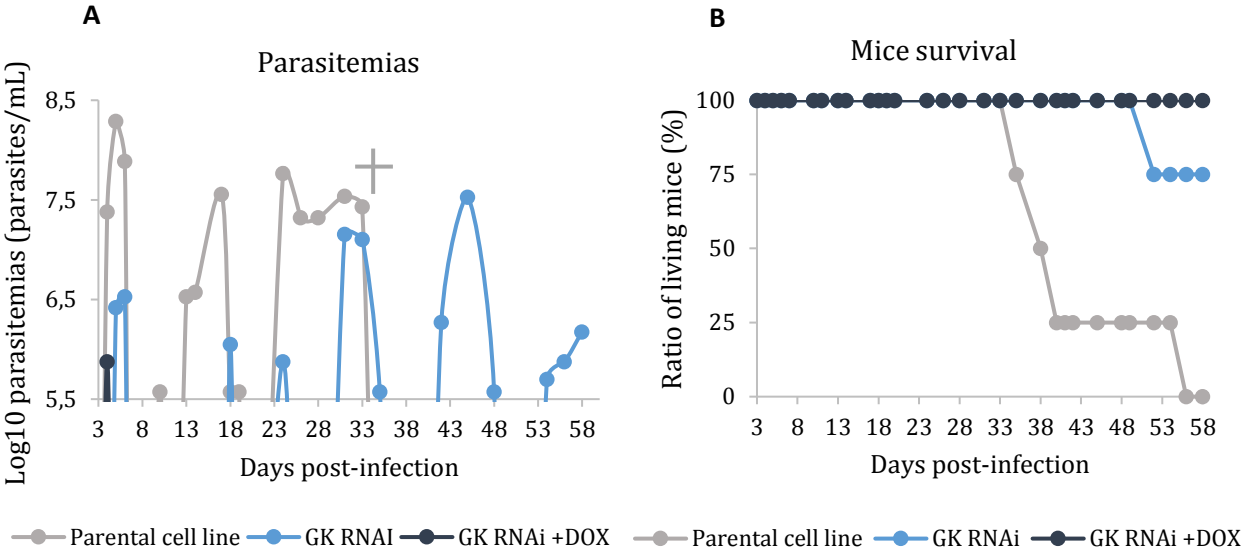

Figure 4.16 – Glycerol kinase depleted *T. brucei* parasites render lower parasitemias and maximize mice survival during infection. (A) – Number of circulating parasites in blood of infected mice determined throughout the time course using a Neubauer chamber. The parasitaemias for all cell lines represented are of individual mice due to heterogeneity (B) – Percentage of living mice for each group throughout the days of infection. ┼ – Mice infected with AnTat 1.1 90:13 GFP-PAD1 parental cell line died earlier from infection than their counterparts infected with the GK RNAi mutant parasite line. Experiment was performed once with four biological replicates per group.

So far, we have demonstrated that glycerol metabolism plays an important role in parasite differentiation to stumpy forms and consequently in regulating and maintaining *T. brucei* infection in mice (**Figure 4.16A)**. This led us to hypothesize that parasites might be using glycerol from its hosts to establish infection and maintain normal levels of parasitemia. Glycerol can be found in the blood of mice at a concentration around 0.3mM[71] derived mainly from lipolysis in the adipose tissue, and also from hydrolysis of triglycerides taking place in plasma lipoproteins[72]. Therefore, we decided to investigate glycerol levels in serum during *T. brucei* infection in mice. To that end, we infect mice with the parental cell line AnTat 1.1 90:13 GFP-PAD1 parasites and quantified glycerolemia at day 6, 9 and 16 post-infection (**Figure 4.17**). As expected, healthy mice showed steady levels of glycerol in their blood throughout the time course, around 0.4 mM, which is consistent with the values in the literature[71]. In contrast, when mice were infected with *T. brucei* parasites, the concentration of glycerol in the blood fluctuated during the infection. On day 6 post-infection, the concentration of glycerol remained similar to non-infected animals. However at day 9, the level of glycerol in the serum decreased to approximately half, indicating that *T. brucei* infection alters glycerol blood concentration of the host.

We propose that *T. brucei* utilizes glycerol throughout infection in mice maybe as an alternative source to glucose and this uptake of glycerol overlaps temporally with peaks of parasite numbers in blood and consequently extensive differentiation of slender to stumpy forms. This is consistent with observation that *T. brucei* metabolizes glycerol *in vitro,* rendering a higher pyruvate yield and stimulating earlier differentiation to stumpy forms.

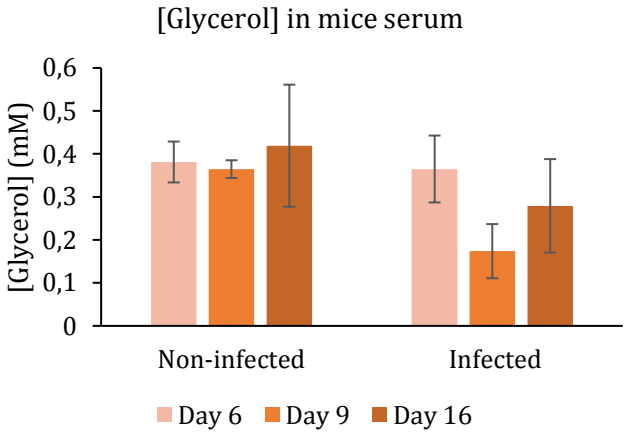

Figure 4.17 – Levels of glycerol in mice serum oscillate during *T. brucei* infection. Glycerol was quantified in the serum at days 6, 9 and 16 post-infection in non-infected mice that served as controls, and mice infected with EATRO 1125 AnTat 1.1 90:13. Experiment was performed once with four to six biological replicates per group. Error bars represent standard deviation from the mean value.

5. Discussion and future perspectives

Since the appropriate conditions enabling axenic culture of pleomorphic BSFs have been established[52], differentiation of slender forms to stumpy forms has been widely studied *in vitro* with the main purpose of elucidating the molecular mechanism and identifying the effector molecule driving this process, termed SIF. This particular step of *T. brucei* life cycle is not only essential for parasite transmission into the insect vector but also vital for mammalian host survival[13]. Therefore, any attempts to impair the slender to stumpy transition would help controlling dissemination of African trypanosomiasis. SIF activity triggers cell cycle arrest in G1/G0 phase and differentiation to stumpy forms through the cAMP signalling pathway of T. *brucei*[12] .cAMP analogues reproduce the SIF activity and induce differentiation to stumpy forms with high efficiency *in vitro*. However, cAMP acts only as a second messenger conveying the SIF signal inside the parasite. Indeed as previous studies have shown, the addition of parasite-conditioned media (containing SIF) causes an increase in cAMP levels inside *T. brucei* pleomorphic cells[12], an effect that is also observed while reaching a parasitemia peak during *in vivo* infection[30]. So far, the chemical identity of SIF remains unknown, apart from a few properties of this compound that are known of common knowledge: SIF is a small molecule (with a molecular mass lower than 500 Da), heat-stable (most likely SIF is not a protein), produced by slender forms which accumulates in the culture medium as parasite density increases.

As bloodstream slender forms rely entirely on glycolysis for their energy production[33], with pyruvate and glycerol being the main end-products, we explored the hypothesis that these metabolites accumulating in culture medium could act as the SIF. We especially focus our efforts on glycerol, as preliminary results from our laboratory showed that this metabolite can induce cell-cycle arrest of parasites in G0/G1 phase associated with the expression of a specific stumpy marker protein, PAD1. In the first part of the results (**section 4.1**), we investigated the efficiency of exogenous glycerol in eliciting parasite differentiation from slender to stumpy forms *in vitro*. We showed that glycerol-induced stumpy cells not only expressed some of the most important molecular markers for stumpy (GFP-PAD1, ESGA9, TAO and GPEET) but were also cell-cycle arrested in G0/G1 phase, besides being functional cells capable to fully differentiate into procyclic insect forms. Interestingly, we showed that glycerol promotes differentiation to stumpy forms in a dose-dependent manner, which is consistent with the mechanism of action of SIF.

Our results show that glycerol is nearly as potent as parasite-derived conditioned medium to induce differentiation to stumpy forms *in vitro*. However, this happens when we add 68 mM of glycerol to the culture medium, which is above the physiological levels (1-2 mM). This evidence could be explained by the additive effect of several molecules present in conditioned medium that have a SIF activity. We assessed previously that even the lowest concentration of glycerol tested

(0,7 mM) promoted a slight increase in insect-transmissible forms in culture comparing to the control condition. The following experiments were performed with the maximal concentration of glycerol (68Mm) in order to better assess the subsequent molecular transformations that accompany slender to stumpy differentiation.

Importantly, we observed that glycerol accumulates in culture medium as parasite density increases, correlating directly with the emergence of quiescent stumpy forms. Interestingly, we showed that differentiation-defective strain of T. brucei, called monomorphic strain, produces even higher amounts of glycerol than pleomorphic cells. Yet, as described for the SIF, monomorphic parasites are able to produce glycerol but do not properly receive and respond to its signal[12]. The effect of glycerol on stumpy formation is dependent on the parasite density in culture. High parasite density induces changes that favour glycerol efficacy on the slender to stumpy transition. Previous studies have linked high parasite density to a decrease in glucose availability[73], moreover, inhibition of glucose transport through THT1 channels induces differentiation to insect-transmissible forms[74]. Oxygen and glucose limitation should result in a decrease of ATP production by the parasites either by switching to anaerobic metabolism or by a decrease of glycolytic flux. Interestingly, treatment with the antibiotic ascofuranone completely eliminated BSFs from infected mice by specifically inhibiting the Trypanosome Alternative Oxidase (TAO)[75], reproducing anaerobic conditions in *T. brucei*. Strikingly, this compound is not only potent to clear parasitemia, but also to induce generation of stumpy forms *in vivo*[76]. Under anaerobic conditions, parasites convert glycerol-3-phosphate into glycerol with the concomitant production of ATP in order to maintain the ATP/ADP ratio balanced inside the glycosome[38,39]. However, anaerobic glucose metabolism in *T. brucei* results in net production of only one ATP per molecule of glucose consumed, rather than two molecules of ATP produced in aerobic conditions. This suggests that anaerobic conditions or/and decrease in net production of ATP could trigger differentiation to stumpy forms of *T. brucei* parasites. Therefore, it would be interesting to measure the ATP production, ADP/ATP and AMP/ATP ratios inside the parasites after exposure to glycerol. We postulate that incubation with glycerol would decrease the ATP production of the parasite and increase AMP/ATP ratio inside the cells. In fact, AMP and hydrolysable derivatives of cAMP ratio have been shown to decrease the activity of *T. brucei* protein kinase target of rapamycin 4 (TbTOR4) and consequently promote differentiation of stumpy-like forms in monomorphic strains[77,31].

Moreover, we showed that glycerol can induce early parasite response, notably, with the regulation of glycolytic genes expression. For example, glucose transporter (THT1), hexokinases (HK1 and HK2) and phosphofructokinase (PFK) RNA transcripts increase after only 6 hours of glycerol exposure. 800µM of exogenous glycerol is sufficient to inhibit 50% of pyruvate formation

in *T. brucei*, at least in anaerobic conditions[78]. Therefore, we hypothesized that parasites upregulate the expression of these glycolytic enzymes in order to compensate for the inhibitory effect of glycerol on the glucose consumption. Glucose consumption could be measured using a commercial kit in order to confirm that *T. brucei* glycolytic flux decreases when parasites are exposed to glycerol. Furthermore, a metabolomics approach coupled with heavy-atom isotope-labelled glycerol could be used to probe the metabolic pathways[58] activated in differentiation to stumpy forms induced by glycerol exposure.

Finally, we showed that co-cultures of parasites with glycerol-producing cells, such as adipocytes, results in premature differentiation to stumpy forms *in vitro*, suggesting again that glycerol production and release in culture medium favours parasite differentiation into stumpy forms. However, it is difficult to know if it is the co-culture that promotes more glycerol production from *T. brucei* parasites or if it is *T. brucei* that stimulates 3T3-L1 fibroblasts to produce and release glycerol. Glycerol is released from adipocytes through a membrane channel called AQP7. Co-cultures of parasites with AQP7 KO adipocytes would aid to understand if glycerol produced by adipocytes are directly responsible for premature differentiation to stumpy forms.

Future experiments, notably, omics approaches such as transcriptomic, proteomics and phosphoproteomics comparing *T. brucei* parasites exposed or not to glycerol will help to not only confirm the nature of these glycerol-induced stumpy forms but also bring new insights into the molecular mechanisms behind differentiation to stumpy forms.

In the second part of the results (**section 4.2**), we aimed to decipher the mechanism by which glycerol induces the slender to stumpy transition of *T. brucei* parasites. To that end, we employed loss-of-function approaches targeting either glycerol production or transport in the parasites. T. brucei utilizes the reverse reaction of glycerol kinase (GK) to produce glycerol[38,39]. *T. brucei* and other african trypanosomes utilize this reverse reaction to produce ATP during anaerobic glycolysis and rescue the parasites from energy starvation when the oxygen utilization in the mitochondrion is compromised. However, the release of glycerol from the cytosol is detrimental for the parasite as high concentrations of this glycolytic end-product will not only inhibit glycerol kinase reverse reaction by mass action, but also decrease ATP production and the glycolytic flux[78,79,80]. Therefore, *T. brucei* has developed mechanisms to transport and secrete glycerol using protein channels termed aquaglyceroporins (AQPs)[44,45]. Therefore, in this project, we decided to block glycerol production or transport in *T. brucei* by depleting either glycerol kinase or the aquaglyceroporin channels, respectively. *T. brucei* genome has 3 different AQP genes which can be knocked-out successfully in slender forms without compromising cell viability[47]. Hence, we created an AQP full KO mutant parasite line with a pleomorphic background and studied its phenotype associated to differentiation to stumpy forms. We confirmed, as described before[47],

that AQP KO parasites are impaired for the transport and secretion of glycerol. As a result, we postulated that parasites defective for glycerol transport would not be able to respond and differentiate to stumpy forms upon culture with either glycerol or parasite-derived conditioned medium. Yet, these parasites show no particular phenotype for differentiation to stumpy forms triggered by either cell density (**Figure 4.B**), glycerol exposure (**Figure 7.1**) or parasite-derived conditioned medium (**Figure 7.2).** These evidences suggest that AQP KO parasites can still sense the SIF signal and uptake glycerol to the cell. In fact, a previous study showed that parasites with a simultaneous knockdown of all 3 AQPs were able to substitute the missing glycerol uptake capability through a putative glycerol transporter[81]. This could explain why our AQP triple KO parasites still respond to glycerol signal and differentiate into stumpy forms at the same extent as wild-type parasites. Future experiments attempting to identify and deplete other putative glycerol transporters would be beneficial to decipher the role of glycerol in differentiation to stumpy forms and *quorum sensing* mechanisms.

Our second approach to understanding the molecular mechanisms behind differentiation to stumpy forms triggered by glycerol was to target directly the production of glycerol by *T. brucei*. Therefore, we depleted the glycerol kinase (GK) activity from *T. brucei* parasites by degraded GK transcripts using RNA interference strategy. As expected, GK depleted parasites did not produce and secrete any glycerol inside the culture medium. However and in contrast to AQP KO cells, GK mutant parasites showed a premature and greater differentiation to stumpy forms than their parental cell line counterparts. Moreover, when conditioned medium from GK depleted parasites (which contains no glycerol) is added to wild-type parasite cultures, cells still enter into a premature differentiation to stumpy forms (**Figure 7.3**).

Therefore and surprisingly, these results suggest that glycerol production and secretion is not essential for differentiation to stumpy forms *in vitro*. Interestingly, GK depleted parasites showed the same phenotype of premature differentiation to stumpy forms as parasites exposed to exogenous glycerol. *T. brucei* parasites can use glycerol as carbon source and convert it into glycerol-3-phosphate inside the glycosome, using glycerol kinase[82]. Additionally, glycerol-3-phosphate can also accumulate inside the glycosome under anaerobic conditions when glycerol kinase is inhibited[39]. Therefore, we propose that premature differentiation to stumpy forms observed with GK depleted parasites and wild-type parasites exposed to glycerol results from intra-glycosomal accumulation of glycerol-3-phosphate. As a result, glycerol-3-phosphate could be a downstream effector that transmits glycerol signal for differentiation to stumpy forms. To test this hypothesis, we could measure the intracellular concentration of glycerol-3-phosphate and its concentration inside the culture medium of GK depleted parasites or parasites exposed to glycerol. More importantly, we could supplement the culture medium of *T. brucei* parasites with

exogenous sn-glycerol-3-phosphate and study any changes for differentiation to stumpy forms *in vitro*.

In the last part of the results section (**section 4.2.5**), we investigated the effect of glycerol metabolism on the dynamics of *T. brucei* infection in mice. The impact of glycerol metabolism in *T. brucei* was studied by infecting mice with parasites that do not produce glycerol (GK depleted parasites). Moreover, the impact of glycerol metabolism from the host was studied by following and measuring variation of glycerol blood concentration during *T. brucei* infection. Interestingly, we showed that mice infected with GK depleted parasites have reduced level of parasitemia throughout the infection associated with 100% mice survival. Indeed, the first peak of parasitemia induced by GK depleted parasites infection is drastically reduced compared to infection with wild-type parasites. Strikingly, all parasites disappear from the mouse blood at day 5 of infection, when extensive transformation of slender forms to stumpy forms normally occurs in infection. It would be interesting to confirm that GK depleted parasites also enter a premature differentiation to stumpy forms in the mouse blood by measuring the expression of the stumpy marker PAD1 coupled to GFP using flow cytometry. Indeed, if GK depleted parasites show premature differentiation to stumpy forms in vivo, this could explain why the first peak of parasitemia is reduced and why parasites are cleared from the mouse blood by day 5, as stumpy forms are non-proliferative forms.

Finally, we followed and measured blood concentration of glycerol during T. brucei infection in mice. Interestingly, we showed that T. brucei infection alters the blood glycerol concentration of mice. Indeed, blood glycerol concentration decrease during day 6 to day 9 of T. brucei infection when differentiation to stumpy forms normally occurs. This result suggests either that T. brucei infection alters the glycerol production of the host or that parasites salvage glycerol from the host to their own usage. First, it would be important to repeat this experiment and to quantify the blood glycerol concentration throughout the mouse infection with more time points. Second, we could study the dynamic of a *T. brucei* infection when mice are deficient for glycerol transport. Indeed, AQP7 KO mice are impaired for the release of glycerol from adipocytes to the bloodstream and consequently display lower glycerol concentrations in their plasma[71]. First, we would like to investigate if this mutation could influence the dynamics of *T. brucei* infection. Secondly, if differentiation to stumpy forms occurs independently from glycerol production of the host. And third, we would like to study if glycerol supplementation in the water supply or injection of glycerol in mice would affect the dynamics of *T. brucei* infection in the host. In this respect, we expect glycerol supplementation to induce a premature differentiation to stumpy forms *in vivo*, similarly to what happens in culture.

Altogether, in this thesis, we have shown that glycerol metabolism in *T. brucei* parasites contributes for the slender to stumpy transition, not only *in vitro* but also in vivo. Moreover, our work revealed that glycerol could be the missing element in the identification of the Stumpy Induction Factor (SIF) in *T. brucei*. Although further experiments are needed, our work brings not only new insights into the molecular mechanisms behind differentiation to stumpy forms but also new avenues for the development of a transmission blocking therapy against Human and Animal African Trypanosomiasis.

6. References

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7. Annexes

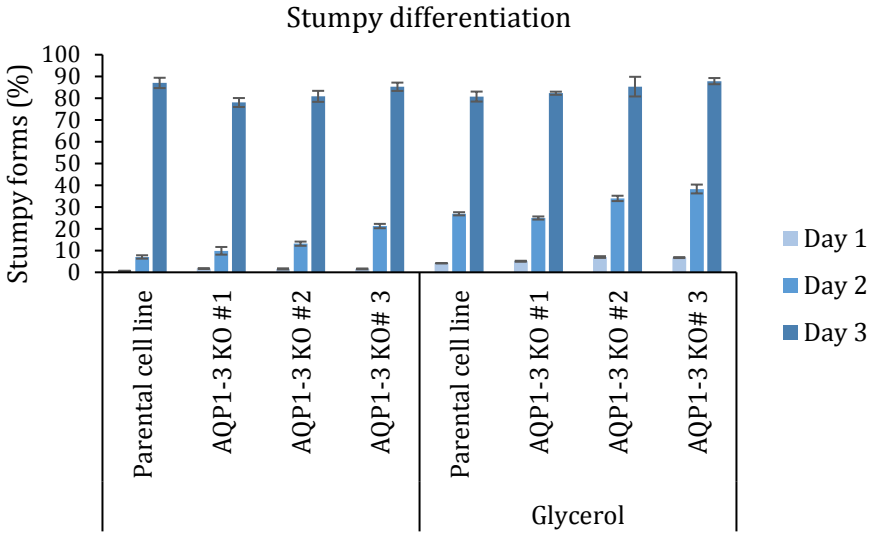

Figure 7.1– Parasites deficient for AQP-mediated transport respond to glycerol by differentiating to stumpy forms. AnTat GFP-PAD1 and AQP1-3 KO parasites were cultured in standard HMI-11 or HMI-11 supplemented with 68 mM glycerol at an initial density of $5x10^4$ parasites/mL, Percentage of stumpy forms in culture was assessed by quantifying GFP-PAD1 expressing cells using flow cytometry. Error bars represent standard deviation from the mean value. Experiment was performed once with three replicates per condition.

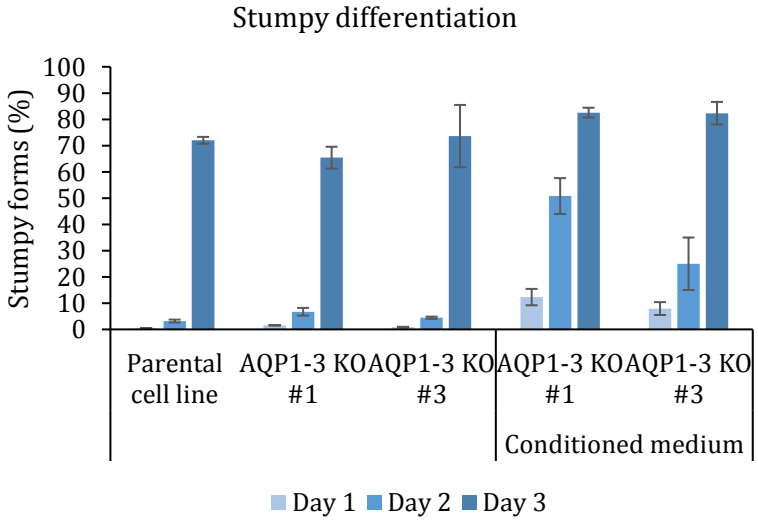

Figure 7.2 – Conditioned medium promoted premature differentiation to stumpy forms of AQP-deficient parasites. AnTat GFP-PAD1 and AQP1-3 KO parasites were cultured with an initial density of $5x10^4$ parasites/mL in standard HMI-11 or HMI-11 supplemented with 50 % of conditioned medium prepared from a pleomorphic strain, Percentage of stumpy forms in culture was assessed by quantifying GFP-PAD1 expressing cells using flow cytometry. Error bars represent standard deviation from the mean value. Experiment was performed one time with two to three biological replicates per condition.

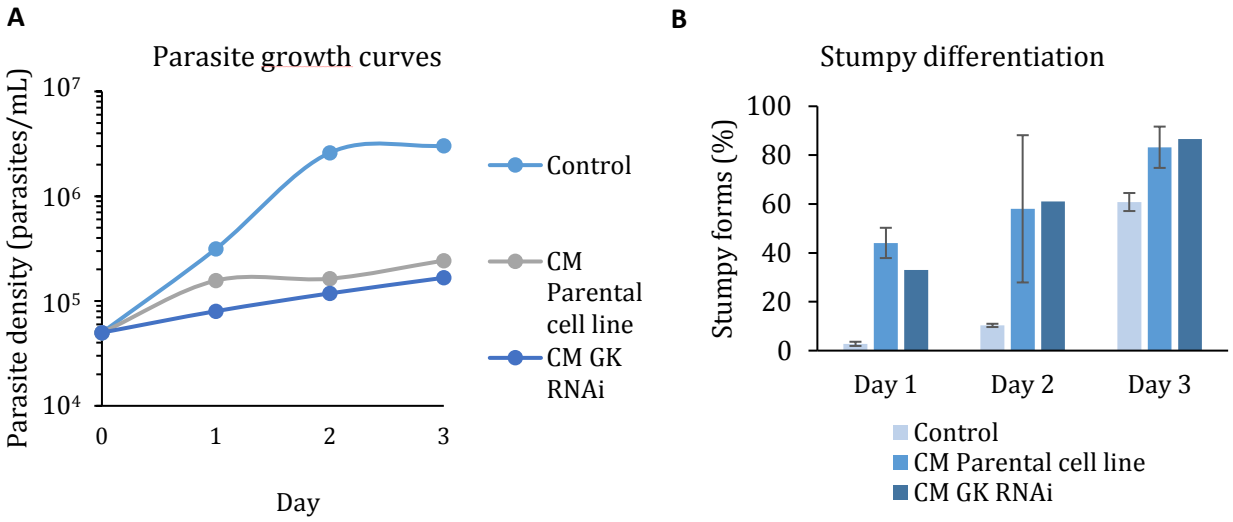

Figure 7.3 – Conditioned medium from glycerol kinase depleted parasites also promotes differentiation to stumpy forms. AnTat 1.1 90:13 GFP-PAD1 (parental cell line) and GK RNAi parasites were cultured with an initial density of $5x10^4$ parasites/mL in standard HMI-11 or HMI-11 supplemented with 50 % of conditioned medium prepared from AnTat 1.1 90:13 GFP-PAD1 parasites or GK RNAi parasites. (A) – Parasite numbers determined daily using a Neubauer chamber. (B) - Percentage of stumpy forms in culture was assessed by quantifying GFP-PAD1 expressing cells using flow cytometry. Error bars represent standard deviation from the mean value. (A-B) - Experiment was performed once with three replicates per condition.