INVESTIGATING METABOLIC PATHWAYS AND PROTEINS IMPORTANT FOR FUELING SPOROZOITE MOTILITY

by

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A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science

Baltimore, Maryland

May 2023

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Abstract

Malaria persists as a major life-threatening disease, owing to the evolution of drug resistant parasites. Our ability to develop new drugs is dependent on the discovery of new parasite-specific biological targets. In general, antimalarial drugs and drug development have primarily focused on blood stage parasites. However, to block malaria transmission, targeting the infective stage of the parasite—sporozoites, is required. A key characteristic of sporozoites is their capacity to perform gliding motility. Without motility, sporozoites would be unable to exit the inoculation site and initiate infection, thereby highlighting the transmission-blocking potential of targeting motility. Like all living organisms, sporozoites need to produce and use energy to move. In this study, we sought to characterize metabolic pathways that are important for producing the energy that fuels sporozoite motility. Using a moderate throughput motility assay, we found that sporozoites can move in the absence of exogenous carbohydrates, and that *P. berghei* and *P. falciparum* motility can be abrogated by both oxidative phosphorylation and glycolysis inhibitors. Moreover, sporozoites express *Plasmodium berghei* vacuolar pyrophosphatase 2 (PbVP2) and vacuolar ATPases (V-ATPases) to pump protons across membranes. However, treatment with vacuolar pyrophosphatase and ATPase inhibitors did not prevent sporozoites from moving. To further elucidate the role and importance of PbVP2, we developed a PbVP2 knockout line. Lastly, we show that motility is significantly inhibited in the presence of fatty-acid free BSA, indicating that fatty acids are also important motility mediators. Our findings bring us closer to understanding sporozoite motility and which metabolic pathways have potential to be targeted in a transmission-blocking approach.

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Introduction

Malaria remains one of the world’s most important infectious diseases, causing 247 million estimated cases across 84 endemic countries in 2021. Malaria’s causative agent are Apicomplexan parasites of the *Plasmodium* species. Infection begins when an *Anopheles* mosquito injects *Plasmodium* parasites into the skin during probing and blood feeding. Injected parasites are in their highly motile sporozoite stage where they use motility to escape the dermis, into the bloodstream, are transported to the liver, infect hepatocytes, and later cause blood-stage infection. Most antimalarial drugs target blood stage parasites and do not have the capacity to block transmission; the development of new transmission-blocking drugs is needed. Because the parasite lifecycle cannot continue if sporozoites are unable to escape the skin, sporozoite motility is a great potential target for preventative intervention with drugs and vaccination. It can take inoculated sporozoites up to 3 h to find and enter into blood vessels. Therefore, the sporozoite stage represents the parasite’s longest extracellular phase within the mammalian host, where it is vulnerable to both innate and adaptive immune responses. As such, metabolic pathways that support gliding motility are essential for parasite survival and the parasite’s ability to cause infection.

Gliding motility is substrate-dependent and relies on several key components including the circumsporozoite protein (CSP), members of the thrombospondin-related adhesive protein (TRAP) family, and a subpellicular actinmyosin motor. Specifically, TRAP proteins are type I transmembrane proteins; their cytoplasmic domains connect with F-actin which binds myosin A, whilst their extracellular domains bind receptors in the matrix or on host cells. Gliding motility involves rapid turnover of these adhesion sites. Bound TRAP-actin complexes become
posteriorly translocated on the parasite surface, consequently pushing the parasite forward in a way similar to how a military tank’s tread would push it forward by translocating backwards\textsuperscript{17–19}. With this adhesion-based gliding mechanism, sporozoites have been observed to move significantly faster than innate immune cells such as neutrophils, at a speed of 1 µm per second or greater\textsuperscript{20}. To achieve this high-speed and sustain motility for hours, sporozoites must produce and spend energy.

Most eukaryotes use ATP as their primary energy source, often produced through two conserved pathways: glycolysis and oxidative phosphorylation\textsuperscript{21}. Glycolysis is a multi-step pathway where glucose is oxidized to produce a net of 2 molecules each of pyruvate, ATP, and NADH. ATP can immediately be used to energize biological processes by harnessing the energy released from the hydrolysis of its phosphodiesterase bond. Cells can reduce pyruvate with NADH to produce lactic acid and NAD\(^+\), where NAD\(^+\) can be reused to fuel additional cycles of glycolysis. Alternatively, in oxidative phosphorylation, pyruvate molecules enter the citric acid cycle where they are oxidized into water and carbon dioxide to produce FADH\(_2\) and more NADH. Then, electrons derived from NADH and FADH\(_2\) are used to reduce oxygen through the mitochondrial electron transport chain. In this process, a proton gradient is generated across inner mitochondrial membranes which is harnessed by ATP synthase to produce additional ATP. Although much more ATP is produced through oxidative phosphorylation compared to a single cycle of glycolysis, \textit{Plasmodium} parasites are known to depend differentially on the two pathways depending on their life cycle stage\textsuperscript{22}.

Previous investigation of \textit{Plasmodium} energy metabolism has largely focused on the parasite’s erythrocytic phases. Blood-stage parasites are avid consumers of glucose; the glucose
consumption rate of infected host erythrocytes can increase up to 100-fold when the parasites transition to metabolically active trophozoite and schizont stages\textsuperscript{23}. Of the glucose consumed, the majority is converted into lactic acid and excreted, whilst very little is oxidized into carbon dioxide\textsuperscript{24}. In addition, \textit{in vitro} cultures of \textit{P. falciparum} exhibit very low oxygen consumption, and morphologically, their mitochondrial cristae are scarcer compared to sporozoites\textsuperscript{25,26}. The knockout of the ATP synthase β-subunit in \textit{P. berghei} and other components of the citric acid cycle does not hinder blood-stage parasite development or their ability to form gametes\textsuperscript{26–28}. Therefore, it has been demonstrated that blood-stage parasites are not dependent on the mitochondrial citric acid cycle, and instead, rely mainly on glycolysis for their ATP production\textsuperscript{26}. This is consistent with the idea that glycolysis is both preferred and sufficient to support rapidly proliferating cells in conditions where glucose is abundant\textsuperscript{29,30}. In comparison, sporozoites exist primarily in changing extracellular environments, and their allocation of energy expenditure may be different\textsuperscript{31}. While intraerythrocytic parasites require energy to fuel growth and proliferation through nucleic acid synthesis and lipid biogenesis, non-proliferative sporozoites must devote energy to fuel gliding motility for cell traversal and invasion\textsuperscript{25,32}.

Studies of sporozoite energy metabolism have used gliding motility as a representation of the parasite’s energy production and consumption\textsuperscript{33}. Unlike blood-stages, \textit{Plasmodium} mosquito stages are thought to rely on oxidative phosphorylation; their expression of enzymes involved in the citric acid cycle is elevated, and oxidative phosphorylation seems to have a critical role in gametocyte maturation, oocyst formation, and oocyst maturation\textsuperscript{34,35}. Only one study conducted 50 years ago examined sporozoite energy metabolism by observing motility\textsuperscript{33}. It showed that inhibition of oxidative phosphorylation with succinate dehydrogenase inhibitors
and membrane uncouplers reduces sporozoite motility. However, inhibitors such as 2,4-DNP
and malonate only showed complete inhibition at high concentrations above 10 mM.
Additionally, several of the used inhibitors such as arsenate and fluoride, are known to have off-
target toxicity extending beyond the ablation of oxidative phosphorylation. As such, it is
unclear as to whether their reduction in motility is caused entirely by the ablation of oxidative
phosphorylation, or through toxicity and/or the inhibition of other biological pathways.

Since then, new antimalarials have been developed and approved for use which can
allow for further investigation. One of them is atovaquone, a competitive inhibitor of
ubiquinone which specifically inhibits the parasite’s mitochondrial electron transport chain at
the bc1 complex. Atovaquone is used for malaria chemoprevention in combination with
proguanil, which synergistically inhibits both liver and blood stage parasites at nanomolar
concentrations. The impact of atovaquone and proguanil on sporozoites has not yet been
investigated, and their effect can help elucidate the role of oxidative phosphorylation in
sustaining motility. Indeed, their specificity for the Plasmodium electron transport chain makes
them excellent inhibitors to test with sporozoites. Furthermore, aside from oxidative
phosphorylation, the role of glycolysis in supporting sporozoite motility also remains unclear.
Although mosquito stage parasites have an increased dependence on oxidative
phosphorylation, they also express glycolytic enzymes. As such, we aimed to investigate
whether gliding motility is dependent on the functioning of one or both pathways.

Sporozoites make an impressive journey from the mosquito midgut to the mammalian
liver, thus must survive and sustain motility in changing environments. Initially, they exit
oocysts in the mosquito midgut to enter the haemolymph from which they recognize and
invade salivary glands. Here they wait, non-motile, in the secretory cavities of the salivary gland until they are injected into the skin of the mammalian host. In the skin they move to locate blood vessels which they enter to be carried by the bloodstream to hepatocytes. Each of these environments have different stressors, resources, and exogenous energy sources which sporozoites must navigate whilst sustaining motility. Previous work to identify the exogenous components required for motility has shown that albumin and calcium are essential. As such, *in vitro* gliding experiments generally use bovine serum albumin (BSA) to stimulate motility. For calcium, motility is initiated through a signaling cascade where intracellular levels of cAMP are elevated and Ca$^{2+}$ is released from the endoplasmic reticulum into the cytoplasm. When intracellular stores of Ca$^{2+}$ are depleted over time, *in vitro* motility can be restored by adding exogenous calcium. Apart from calcium and albumin, the reintroduction of carbohydrates and amino acids including glucose, trehalose, alanine, asparagine, and glutamine has been shown to partially restore motility in media without other exogenous energy sources. Thus, sporozoites are also thought to be dependent on exogenous carbohydrates and amino acids to sustain motility.

Pyrophosphate (PP$_i$) is another molecule whose energy can be harnessed to drive biological pathways. Because pyrophosphate can spontaneously form in conditions that supported early prokaryotic life, it is thought have been the primary energy source of organisms prior to ATP. Even today, pyrophosphate is produced by hundreds of intracellular reactions where nucleoside triphosphates such as ATP and GTP are hydrolyzed into nucleoside monophosphates and a PP$_i$. The energy released in this reaction is 35 kJ/mol and is derived from the energy stored in phosphodiester bonds; when hydrolyzed, the energy from the bond...
can be coupled to drive enzymatic reactions. Like a nucleoside triphosphate, the phosphodiester bond in PP$_i$ can also be harnessed as energy; the hydrolysis of a single PP$_i$ can yield a comparable 20-25 kJ/mol of energy. Because PP$_i$ can exert negative feedback on reactions that produce it, its concentration in the cell is regulated by pyrophosphatases (PPases). PPases are enzymes which couple the hydrolysis of PP$_i$ to catalyze reactions; they have been evolutionarily conserved in plants, protozoans, bacteria, and archaea.

A subcategory of PPases that use energy from the hydrolysis of PP$_i$ to pump protons across biological membranes are known as vacuolar-type proton pumping pyrophosphatases, or V-type H$^+$.PPases. *Plasmodium* parasites are known to encode two V-type H$^+$.PPases; *P. falciparum* encodes PfVP1 (Pf3D7_1456800) and PfVP2 (Pf3D7_1235200), which are homologous to PbVP1 (PBANKA_1320500) and PbVP2 (PBANKA_1449800) in *P. berghei*. In blood stage parasites, knockout of PfVP1 delays ring stage development and completely blocks development to the trophozoite stage. While PfVP1 and PbVP1 are highly expressed in blood stages, PfVP2 and PbVP2 are expressed in oocyst and salivary gland sporozoites. Although essential for ring stage parasites, the role of V-type H$^+$.PPases in sporozoites remains unknown.

Separate from V-type H$^+$.PPases, vacuolar-type ATPases (V-ATPases) are essential ATP-driven proton pumps, evolutionarily conserved in eukaryotic organisms. Through the coupling of ATP hydrolysis to pump protons across intracellular and plasma membranes, V-ATPases are important regulators of vacuolar acidification and cytosolic pH homeostasis. In *P. falciparum* blood-stage parasites, V-ATPases have been shown to localize to the plasma membrane and pump protons out of the cell to acidify the nearby environment within the cytosol of infected erythrocytes. In addition, inhibition of V-ATPase-dependent lysosomal acidification has been
shown to block *P. yoelli* sporozoite egress from transient vacuoles during invasion of HepG2 liver cells. Like V-type H^+-PPases, whether V-ATPase function is important in sustaining gliding motility remains unclear. In this study, we tested motility in the presence of the selective V-ATPase inhibitors, bafilomycin A1 and concanamycin A, to determine if motility is dependent on V-ATPase function.

Fatty acids represent another class of nutrients that are necessary for the survival of *Plasmodium* parasites. In general, fatty acids are important for many biological processes including membrane formation, signalling, and energy production. To obtain them, malaria parasites can either scavenge exogenous fatty acids from their environment or synthesize them via the FAS-II biosynthesis pathway in their apicoplast. Enzymes of this pathway are expressed primarily in mosquito and liver stage parasites; knockout of FAS-II genes prevents proper *P. falciparum* sporozoite development and *P. yoelii* liver stage progression. For many mammalian cells, fatty acids are used as an energy source through the pathway of β-oxidation. Through β-oxidation, fatty acids are catabolized into substrates of the citric acid cycle, thereby contributing to downstream ATP production through oxidative phosphorylation. Although mosquito-stage parasites rely on a functional citric acid cycle, functional β-oxidation in *Plasmodium* is lacking. Specifically, multiple enzymes of the β-oxidation pathway, including carnitine palmitoyl transferase, which conventionally initiates fatty acid oxidation in mitochondria, are missing in *Plasmodium*. As such, it is unlikely that energy for sporozoite motility is produced through β-oxidation. Whether fatty acids play a role in facilitating sporozoite motility remains unknown.
The overall aim of this study was to identify metabolic pathways responsible for producing the energy that fuels sporozoite gliding motility. First, we investigated the importance of glucose in sustaining motility by observing differences in motility in media with or without glucose. Next, we treated sporozoites with atovaquone and proguanil to elucidate the importance of oxidative phosphorylation in sustaining motility. Additionally, we evaluated the importance of glycolysis by observing motility after treatment with the glycolysis inhibitor 2-Deoxy-D-glucose (2-DG). We also investigated the role of V-type H\(^+\)-PPases and V-ATPases in supporting sporozoite motility through treatment with pyrophosphatase inhibitors and the selective V-ATPase inhibitors: bafilomycin A1 and concanamycin A. To further understand the importance of V-type H\(^+\)-PPases, we produced a PbVP2 knockout line. Finally, we explored whether sporozoite motility is dependent on fatty acids by observing motility in media with fatty acid free BSA.

Materials and Methods

Chemicals and Media

Atovaquone (catalog number B2078; ApexBio), concanamycin A (catalog number sc-20211A; Santa Cruz Biotechnology), and bafilomycin A (catalog number 11038; Cayman Chemical Company) were purchased and dissolved in dimethyl sulfoxide (DMSO; Sigma) and stored in -20°C. 2-Deoxy-D-glucose (catalog number D8375-1G) was purchased from Sigma and stored at 4°C; 10 mM stocks were dissolved for use either in Hank’s balanced salt solution (HBSS; catalog number 14025092; ThermoFisher Scientific) or HBSS made without glucose. Pamidronate (catalog number B1807; ApexBio) was purchased and stored at in -20°C after
dissolving in H$_2$O. Imidodiphosphate (IDP) sodium salt (catalog number sc-501001; Santa Cruz Biotechnology) was stored at 4°C and dissolved in H$_2$O for use. Proguanil hydrochloride was provided by Rahul Bakshi, dissolved in H$_2$O, and stored in -20°C. Aminomethylene disphosphonate (AMDP) was provided by Silvia NJ Moreno from the University of Georgia and dissolved for use either in HBSS or HBSS made without glucose. The formulation of HBSS without glucose used in our experiments is listed in Supplementary table 1; it has the same formulation as HBSS (catalog number 14025092; ThermoFisher Scientific) formed by adding each salt component into Milli Q H$_2$O excluding D-glucose. Other media include Dulbecco’s modified eagle medium (DMEM; catalog number 10313021; ThermoFisher Scientific), RPMI; catalog number 21870076; ThermoFisher Scientific), and 10x PBS (catalog number 70011044; ThermoFisher Scientific) which was diluted to 1x in H$_2$O for use.

Dialysis of BSA

To test motility in dialyzed BSA, 3 mL of 30% BSA solution (catalog number BSA-30-0050; Rockland) was injected into a pre-hydrated 3 mL Slide-A-Lyzer™ dialysis cassette (10K MWCO, catalog number 66382; ThermoFisher Scientific). The filled cassette was dialyzed at 4°C in 500 mL of lab made HBSS without glucose. Buffer was changed every 2 hours twice, left overnight, and changed again in the morning for another 2 hours before the dialyzed BSA sample was extracted for use.

Generation of PbVP2 Knockout Plasmid

A PbVP2 knockout vector (PBANKA_144980; vector design ID PbGEM-257126) was designed and provided through the PlasmoGEM project at the Sanger Institute in the U.K. and
Umea University in Sweden. The provided vector was constructed using a long 14kb pJAZZ-OK NotI vector backbone from Lucigen, derived from bacteriophage N15. The vector was provided in an *E. coli* agar stab; it is a linear low copy number vector that replicates in *E. coli* with its encoded *telN* telomerase gene. Complete details about vector design and production are outlined by *PlasmoGEM*\textsuperscript{70}. An overview vector backbone and insert components are shown in figure 1.

![Plasmid map of PbVP2 knockout vector backbone and insert. The pJAZZ-OK backbone contains the phage genes *telN* and *repA*, and cB sequence required for replication. Genes of interest are inserted in the multiple cloning site (MCS) and the remaining backbone has a kanamycin selection factor (Kan). The length of the backbone is 13.4kb, and the length of the knockout insert is 7.9kb. On the insert, left and right recombination arms are homologous to wild-type PBANKA_144980 encoding the V-type-H\(^+\)-PPase VP2; successful insertion will replace the functional gene with this insert. The human dihydrofolate reductase (hDHFR) and yfcU genes are present as positive and negative selection markers respectively. The EF-1 alpha promoter enables transcription of both. The insert also has a 3xHA tag and repeats of PbDHFR terminator sequence.](image)

Figure 1. Plasmid map of PbVP2 knockout vector backbone and insert. The pJAZZ-OK backbone contains the phage genes *telN* and *repA*, and cB sequence required for replication. Genes of interest are inserted in the multiple cloning site (MCS) and the remaining backbone has a kanamycin selection factor (Kan). The length of the backbone is 13.4kb, and the length of the knockout insert is 7.9kb. On the insert, left and right recombination arms are homologous to wild-type PBANKA_144980 encoding the V-type-H\(^+\)-PPase VP2; successful insertion will replace the functional gene with this insert. The human dihydrofolate reductase (hDHFR) and yfcU genes are present as positive and negative selection markers respectively. The EF-1 alpha promoter enables transcription of both. The insert also has a 3xHA tag and repeats of PbDHFR terminator sequence.

**Transfection of PbVP2 Knockout Plasmid into P. berghei WT-ANKA parasites**

To prepare *PbVP2* knockout plasmid DNA for transfection, plasmid-expressing *E. coli* from agar stabs provided by PlasmoGEM were grown on kanamycin positive (30 µg/mL) terrific broth (TB) agar plates. Colonies were then selected and 5 mL liquid cultures (Terrific Broth (TB) medium with 0.4% (vol/vol) glycerol) were inoculated and grown overnight at 37°C shaking at
200 rpm. A larger 250 mL culture was inoculated the following afternoon with 100 µL of the initial culture. After growth overnight, DNA was purified with a midiprep kit (catalog number 12143; Qiagen). To confirm the presence of an unmodified target locus, the plasmid was sanger sequenced using primers listed in Supplementary Table 1, and digested with NotI to detect expected bands. Before transfection, *PbVP2* vector DNA was digested with NotI overnight at 37°C. Then, 10 µg of plasmid DNA was electroporated into 1.3 × 10⁸ *P. berghei* schizonts and immediately injected intravenously into a Swiss Webster mouse (Taconic). After 24 h, the mouse was given water containing pyrimethamine (70 µg/mL). After 5 days, the pyrimethamine water was removed, and blood was collected when parasitemias reached ~4%. Collected blood was frozen for storage or used for gDNA extraction.

**Infection of Mosquitoes with *P. berghei* WT-ANKA and *PbVP2* KO parasites**

Swiss Webster mice (Taconic) were injected intraperitoneally with *P. berghei* ANKA wild-type parasites or *PbVP2* knockout parasites. Once parasitemia reached around 2%, the mice were bled by cardiac puncture, and blood was diluted in RPMI to 0.01% parasitemia and 200 µL was intravenously inoculated into 3 to 4 mice. Once gametocytemia of 0.1-0.3% was observed, *A. stephensi* mosquitoes (2 days after pupation) were allowed to feed on the mice. Thereafter, mosquitoes were provided with a 10% (wt/vol) sucrose solution and kept at 18°C with 80% humidity. At day 14 post-feeding, mosquito midguts were dissected and stained with 0.1% mercuriochrome in PBS to count oocysts. At days 18-23 post-feeding, mosquito salivary glands were dissected and sporozoite load was measured using a haemocytometer. Mosquito dissection for all experiments was conducted in either HBSS, or in HBSS without glucose for motility assays testing inhibitors in glucose-scarce conditions.
Testing Compounds against P. berghei and P. falciparum Sporozoite Motility

Observation and quantitative comparison of sporozoite motility was conducted with the use of a moderate-throughput motility assay previously described\(^1\). Single 12 mm coverslips were placed into 24-well plates, where each coverslip was pre-washed with MilliQ water and 100% ethanol. Thereafter, wells were filled with 500 µL of CSP-binding antibody in PBS (mAb 3D11 for *P. berghei* or mAb 2A10 for *P. falciparum* sporozoites) and left at room temperature overnight. The following day, into each coverslip, a total of 50,000 sporozoites were added and mixed with compounds of interest or 1 µM cytochalasin D in selected media with 1% (wt/vol) BSA (pH 7.4) in 1.5 mL tubes and incubated for 30 min at 20°C. Selected media includes HBSS, DMEM, RPMI, and HBSS without glucose. After incubation, mAb 3D11/2A10-coated wells were washed twice with PBS and each sporozoite-compound mixture was added into separate wells, centrifuged onto the coverslips for 5 min at 150 x \(g\), and then incubated for 1 h at 37°C and 5% CO\(_2\). Subsequently, the sporozoite-compound mixtures were carefully removed, and remaining bound sporozoites were fixed onto coverslips with 4% paraformaldehyde (PFA) in PBS for 1 h, and blocked with 1% (wt/vol) BSA in PBS (pH 7.4) at room temperature for 30 min. Next, the sporozoites and their CSP trails were stained through the addition of biotinylated mAb 3D11 or biotinylated mAb 2A10 diluted 1:500 in in PBS (pH 7.4) 1% (wt/vol) BSA for 1 h at room temperature. Lastly, wells were washed and incubated with Alexa Fluor 488 streptavidin (Invitrogen) diluted at 1:500 in PBS for 1 h at room temperature. Coverslips were mounted on gold antifade reagent (catalog number P36935; Invitrogen) on glass slides, and imaged by fluorescence microscopy (Nikon E600) with either a 40x or 20x objective lens. Per coverslip, 25-50 fields were imaged under the same exposure. The fluorescence intensity of the images were
quantified using batch processing via ImageJ (Supplementary Figure 1), and compared with graphical representation on GraphPad Prism.
Results

*Testing P. berghei and P. falciparum sporozoite motility in the absence of an exogenous carbon source*

We began by testing sporozoite motility in different physiological buffers to observe their ability to sustain motility in the absence of carbohydrates and amino acids. HBSS, DMEM, and RPMI were used as nutrient rich buffers. HBSS is a salt solution with 5.56 mM of glucose as the only added carbon source, whereas DMEM and RPMI formulations also contain amino acids and sodium pyruvate. To observe motility in the absence of external carbon sources, we made our own HBSS with the same formulation as HBSS provided by Invitrogen, except without any added glucose (Supplementary Table 2). Since sporozoites are thought to rely on glycolysis and/or oxidative phosphorylation for ATP production, we hypothesized that motility would be greatly, if not completely abrogated in the absence of glucose. To test this, we conducted a gliding assay in the different media for 1 h. Imaging and comparison of motility in each condition is shown in Figure 2, where we observed that for both *P. berghei* and *P. falciparum*, the absence of glucose in HBSS did not affect sporozoite motility when compared to motility in media with glucose. Overall, the absence of an exogenous carbon source did not hinder sporozoite motility. Since HBSS without glucose does not contain other potential energy sources such as pyruvate, amino acids, or fatty acids, these results suggested that sporozoites can sustain motility using exogenous energy sources scavenged whilst in the salivary gland and/or with endogenous energy sources.
Figure 2. Testing *P. berghei* and *P. falciparum* sporozoite motility in the absence of an exogenous carbon source. (A&B) Images of *P. berghei* (A) and *P. falciparum* (B) CSP-stained sporozoites and motility trails. Sporozoites were preincubated in DMEM, RPMI, HBSS, HBSS without glucose, or in HBSS with cytochalasin D (1µM) for 30 min and then allowed to move on coverslips for 1 h in the same respective conditions. CSP on sporozoites and their shed trails...
were stained, and images were captured with a Nikon E600 microscope using a 40x and 20x objective lens for *P. berghei* and *P. falciparum* respectively. (C and D) Measurement and comparison of the fluorescence intensities of CSP-stained sporozoites and trails in each *P. berghei* (C) and *P. falciparum* (D) treatment group. Each dot represents the fluorescence intensity of one image, and 60 images were taken for each treatment group (30 per biological replicate). The fluorescence intensity of each condition was compared to each other (****, *P* < 0.0001 [by a Kruskal-Wallis test followed by Dunn’s test]). Red bars represent the mean of each group.

**Testing motility in the absence of glucose with dialyzed BSA**

Although motility was sustained in HBSS without glucose, those conditions contained BSA. Because albumin is a carrier molecule, we hypothesized that small molecules carried by BSA, potentially carbohydrates, fatty acids, or amino acids, were being used as energy sources. To remove these molecules from the BSA, 30% BSA solution was dialyzed in HBSS without glucose. Then, sporozoite motility was observed in HBSS with or without glucose with either dialyzed BSA or non-dialyzed BSA. We found that there was no significant difference in motility between groups supplemented with non-dialyzed BSA and dialyzed BSA (Figure 3B). Sporozoites still demonstrated full motility in HBSS with or without glucose, regardless of whether dialyzed or undialyzed BSA was used (Figure 3A).
Figure 3. Testing *P. berghei* sporozoite motility with dialyzed BSA in HBSS with or without glucose. (A) Images of *P. berghei* CSP-stained sporozoites and motility trails from each treatment group. Sporozoites were preincubated for 30 min in HBSS with or without glucose, or in HBSS with cytochalasin D (1µM) with either dialyzed or non-dialyzed BSA (1% wt/vol).
Following this, sporozoites were allowed to move on coverslips for 1 h in the same respective conditions. (B) Measurement and comparison of the fluorescence intensities of CSP-stained sporozoites and trails in each treatment group. Each dot represents the fluorescence intensity of one image, and 60 images were taken for each treatment group (30 per replicate well). The fluorescence intensity of each condition was compared to each other (****, P < 0.0001 [by a Kruskal-Wallis test followed by Dunn’s test]). Red bars represent the mean of each group.

**Oxidative phosphorylation is important for P. berghei and P. falciparum sporozoite motility in the absence of an external carbon source**

Next, we investigated whether sporozoites require oxidative phosphorylation to sustain motility in the presence and absence of an external carbon source. After observing that sporozoites can move in the absence of exogenous glucose, we hypothesized that sporozoites might rely on the citric acid cycle to fuel motility, as it’s overall output of ATP per glucose molecule is greater than that of glycolysis. Thus, we expected that inhibition of the electron transport chain would abrogate motility both in the presence of an external glucose source, and more severely in the absence. To investigate this, sporozoites were allowed to move in media with different concentrations of the *Plasmodium*-specific mitochondrial electron transport inhibitors: atovaquone and proguanil. Because atovaquone is hydrophobic and dissolved in dimethyl sulfoxide (DMSO), motility in HBSS with 1% (wt/vol) BSA either with or without glucose containing 0.1% DMSO were used as positive controls. As shown in Figure 4, the inhibition of mitochondrial electron transport with up to 100 µM of atovaquone and proguanil did not impact motility within glucose-rich media. By contrast, we observed significant inhibition of both *P. berghei* and *P. falciparum* motility when electron transport was inhibited in
conditions without glucose. Overall, treatment with atovaquone and proguanil only inhibited motility in conditions without an exogenous carbon source.
**Figure 4.** *P. berghei* and *P. falciparum* sporozoite motility after treatment with atovaquone and proguanil in the presence or absence of an exogenous carbon source. (A) Images of *P. berghei* and (B) *P. falciparum* CSP-stained sporozoites and motility trails. Sporozoites were preincubated in HBSS 0.1% DMSO with or without glucose alone, with added Atovaquone and Proguanil at the indicated concentrations, or in HBSS with 1µM cytochalasin D. Following this, sporozoites were allowed to move on coverslips for 1 h in the same respective conditions. CSP on sporozoites and their shed trails were stained, and images were captured with a Nikon E600 microscope using a 40x and 20x objective lens for *P. berghei* and *P. falciparum* respectively. (C and D) Measurement and comparison of the fluorescence intensities of CSP-stained sporozoites and trails in each (C) *P. berghei* and (D) *P. falciparum* treatment group. Each dot represents the fluorescence intensity of one image, and 60 images were taken for each treatment group (30 per replicate well). The fluorescence intensity of each condition was compared to each other (****, P < 0.0001 [by a Kruskal-Wallis test followed by Dunn’s test]). Red bars represent the mean of each group.

**Little glucose is required to support *P. berghei* sporozoite motility with atovaquone & proguanil**

After observing the inhibition of sporozoite motility by atovaquone and proguanil exclusively in glucose-free conditions, we sought to determine how much glucose is needed to overcome this inhibition. To investigate this, sporozoites were allowed to move in HBSS 1% (wt/vol) BSA with 10 µM atovaquone and proguanil and decreasing concentrations of glucose. Again, because atovaquone is dissolved in DMSO, motility in HBSS with 1% (wt/vol) BSA either with or without glucose containing 0.1% DMSO were used as positive controls. Remarkably, we observed that sporozoites remained fully motile with as low as 1 µM of glucose in the presence of 10 µM atovaquone and proguanil (Figure 5). At 0.1 µM of glucose and lower, sporozoite motility was completely inhibited by atovaquone and proguanil (Figure 5). Overall, very little exogenous glucose is needed to overcome the atovaquone+proguanil-mediated inhibition of oxidative phosphorylation and motility.
Figure 5. Testing *P. berghei* sporozoite motility with 10 µM atovaquone and proguanil in different concentrations of glucose. (A) Images of *P. berghei* (CSP-stained sporozoites and
motility trails. Initially for 30 minutes, sporozoites were preincubated in HBSS with decreasing concentrations of glucose, or in HBSS with cytochalasin D (1µM). Then, sporozoites were allowed to move on coverslips for 1 h in the same respective conditions. CSP on sporozoites and their shed trails were stained, and images were captured with a Nikon E600 microscope using a 40x objective lens. (B) Measurement and comparison of the fluorescence intensities of CSP-stained sporozoites and trails in each treatment group. Each dot represents the fluorescence intensity of one image, and 60 images were taken for each treatment group (30 per replicate well). The fluorescence intensity of each condition was compared to each other (****, \( P < 0.0001 \) [by a Kruskal-Wallis test followed by Dunn’s test]). Red bars represent the mean of each group.

Is glycolysis is required for \( P. \) berghei and \( P. \) falciparum sporozoite motility?

Seeing that sporozoites can still sustain motility when oxidative phosphorylation is inhibited in the presence of exogenous glucose, we next sought to investigate whether glycolysis is the pathway by which they do so. Also, because blood-stage parasites function without a functional citric acid cycle, and rely primarily on glycolysis to generate ATP, we thought that sporozoite metabolism may also rely on glycolysis. We hypothesized that sporozoite motility would be impacted by inhibition of glycolysis. We also wondered how sporozoites were obtaining their energy in the absence of exogenous glucose. Were they still dependent on glycolysis? To investigate this, we used 2-deoxy-D-glucose (2-DG). 2-DG has a near identical molecular structure to glucose where the 2-hydroxyl group is replaced with a hydrogen. Consequently, 2-DG can be taken up by glucose transporters and act as a competitive inhibitor of the second step of glycolysis; it prevents the conversion of glucose-6-phosphate into fructose-6-phosphate by the enzyme phosphoglucoisomerase. \( P. \) berghei and \( P. \) falciparum sporozoites were preincubated for 30 min and allowed to move for 1h in media with increasing concentrations of 2-DG in HBSS with 1% (wt/vol) BSA, either with glucose (5.56 mM) or without. Because of its similar structure to glucose, we treated sporozoites with up to 10
mM of 2-DG such that there is enough inhibitor to compete with the 5.56 mM of glucose in HBSS. In glucose-rich HBSS, we found that 2-DG significantly inhibited *P. falciparum* motility at 10 mM, although not completely. However, for *P. berghei*, 2-DG did not inhibit motility in glucose-rich HBSS at concentrations up to 10 mM of 2-DG (Figure 6). By contrast, in HBSS without glucose, complete inhibition of *P. falciparum* motility was observed at each concentration of 2-DG from as low as 100 µM. For *P. berghei*, motility was partially inhibited at 100 µM and completely at 1 mM (Figure 6).
B

**P. berghei**

Fluorescence Intensity (AU)

+/- Glucose

1µM 100µM 1mM 10mM 100µM 1mM 10mM

CytoD 2-DG

C

**P. falciparum**

2-DG Concentration: 100 µM 1 mM 10 mM

+Glucose

-Glucose
**Figure 6.** Testing *P. berghei* and *P. falciparum* sporozoite motility in treatment with 2-Deoxy-D-glucose (2-DG) in the presence or absence of an exogenous carbon source. **(A and C)** Images of *P. berghei* (A) and *P. falciparum* (C) CSP-stained sporozoites and motility trails. Initially for 30 minutes, sporozoites were preincubated in HBSS with or without glucose alone, with added 2-DG at different concentrations (100 µM, 1 mM, and 10 mM), or in HBSS with cytochalasin D (1µM). Then, sporozoites were allowed to move on coverslips for 1 h in the same respective conditions. CSP on sporozoites and their shed trails were stained, and images were captured with a Nikon E600 microscope using a 40x and 20x objective lens for *P. berghei* and *P. falciparum*, respectively. **(B and D)** Measurement and comparison of the fluorescence intensities of CSP-stained sporozoites and trails in each **(B) P. berghei** and **(D) P. falciparum** treatment group. Each dot represents the fluorescence intensity of one image, and 150 images were taken for each treatment group (50 per replicate well). The fluorescence intensity of each condition was compared to each other (****, *P* < 0.0001 [by a Kruskal-Wallis test followed by Dunn’s test]). Red bars represent the mean of each group.
Investigating if proton pyrophosphatase (V-type H\textsuperscript{+}-PPase) activity is important for \textit{P. berghei} sporozoite motility

Apart from ATP produced through glycolysis and oxidative phosphorylation, we investigated if PP\textsubscript{i} dependent pathways are important for motility. After seeing that sporozoites sustain motility in the absence of exogenous glucose, we thought that PP\textsubscript{i} could represent an alternative energy source. Because sporozoites express a V-type H\textsuperscript{+}-PPase which harnesses energy from PP\textsubscript{i} hydrolysis to fuel proton transport across vacuolar and plasma membranes, we hypothesized that sporozoite motility would be abrogated if this V-type H\textsuperscript{+}-PPase is inhibited.

To investigate this, \textit{P. berghei} sporozoites were preincubated for 30 min and allowed to move for 1h in media containing increasing concentrations of the PP\textsubscript{i} analogues aminomethylene bisphosphonate (AMDP) or pamidronate, or the PP\textsubscript{i} hydrolysis inhibitor imidodiphosphate (IDP). The effective concentrations of AMDP, pamidronate, and IDP were in ranges that work to inhibit \textit{Toxoplasma}, plant, and trypanosomatid V-type H\textsuperscript{+}-PPase activity. Motility was observed in HBSS with 1% (wt/vol) BSA, either with glucose (5.56 mM) or in the absence of glucose. After treatment with each compound, in both glucose rich HBSS and HBSS without glucose, we observed no inhibition of sporozoite motility (Figure 7).
Figure 7. Testing *P. berghei* sporozoite motility after treatment with AMDP, pamidronate, and IDP in the presence or absence of glucose. Images of *P. berghei* CSP-stained sporozoites and motility trails from AMDP-treated (A), pamidronate-treated (B), and IDP-treated (C) sporozoites. Initially for 30 minutes, sporozoites were preincubated in HBSS with or without glucose alone, with added AMDP, pamidronate, or IDP at different concentrations (100 µM, 1 mM, and 10 mM), or in HBSS with cytochalasin D (1µM). Then, sporozoites were allowed to move on coverslips for 1 h in the same respective conditions. CSP on sporozoites and their shed trails were stained, and images were captured with a Nikon E600 microscope using a 40x objective lens. Measurement and comparison of the fluorescence intensities of CSP-stained sporozoites and trails in each (A) AMDP, (B) pamidronate, and (C) IDP treatment group. Each
dot represents the fluorescence intensity of one image, and 150 images were taken for each treatment group (50 per replicate well). The fluorescence intensity of each condition were compared to each other (****, \( P < 0.0001 \) [by a Kruskal-Wallis test followed by Dunn’s test]). Red bars represent the mean of each group.

**Confirmation of PbVP2 KO Parasite Clones**

Although treatment with the pyrophosphatase inhibitors AMDP, IDP, and pamidronate did not affect sporozoite motility, it is unclear whether they successfully penetrated and inhibited the intended H-PPase VP2 due to their highly charged nature. Therefore, to further investigate the role of H-PPases in sporozoites, we created PbVP2 knockout parasites. We transfected a PbVP2 knockout plasmid (Figure 1) into wildtype *P. berghei* schizonts. Schizonts were i.v. injected into a mouse which was then subject to pyrimethamine-based positive selection for successful transfectants. Blood was isolated post-selection and used for the cloning in 20 mice. By day 7 post-injection, 3 clones were positive and blood containing 5-6% parasitemia was collected. PCR was conducted on gDNA isolated from the blood of each clone with primers to detect successful 3’ integration (2.2kb expected band) or the presence of wildtype PbVP2 (0.72 kb expected band) (Figure 8A). Wildtype *P. berghei* DNA was used as control, where we observed the absence of our knockout insert (no observable 2.2kb band), but the presence of wildtype PbVP2 (observable 0.72kb band) (Figure 8B). For clone 1, we detected the expected 2.2kb band indicating the presence of parasites containing our knockout insert; however, we also detected a 0.72kb band indicating that the clone also contains wildtype parasites (Figure 8B). For clone 2 and 3, we only detected a 2.2kb band, demonstrating that these clones contain PbVP2 knockout parasites without wildtype contamination (Figure 8B). As such, we used the blood from these clones to conduct our mosquito cycles.
Figure 8. PbVP2 KO Confirmation (A) Diagram of PbVP2 KO insert and wildtype DNA; GW2 + GT primers and QCR1 + QCR2 primers were used to detect 3' insert confirmation and wildtype parasites respectively. A detailed depiction of the components of the KO Cassette are shown in Figure 1 (B) Confirmation gel of PbVP2 knockout parasites; PCR fragments were run on a 0.8% agarose gel for 1.5 h at 80V. For each clone, one lane contains 3’ confirmation fragments, while the other contains wildtype fragments. DNA from wildtype *P. berghei* was used as a control. Primer sequences are listed in Supplementary Table 1.
Testing if Vacuolar $H^+\text{-ATPase}$ Inhibition Affects $P. berghei$ Sporozoite Motility

Apart from pyrophosphate driven $H^+\text{-PPases}$, V-ATPases are vacuolar ATP-driven proton pumps that mediate lysosomal acidification and pH regulation. The maintenance of pH homeostasis is necessary for the functioning of many metabolic pathways. As such, we hypothesized that V-ATPase function could play a role in mediating sporozoite motility; loss of pH homeostasis may hinder energy production pathways or intracellular signalling required for gliding. To test this, we treated sporozoites with either bafilomycin A1 or concanamycin A, both selective inhibitors of V-ATPase mediated lysosomal acidification. Since this process is ATP-driven, we also hypothesized that any hinderance of sporozoite motility would be more severe in media without an exogenous carbon source. As such, $P. berghei$ sporozoites were preincubated for 30 min and allowed to move for 1h in media containing 10 µM or 100 µM of either bafilomycin A1 or concanamycin A. Because both inhibitors are hydrophobic and dissolved in DMSO, motility in HBSS with 1% (wt/vol) BSA either with or without glucose containing 0.1% DMSO were used as positive controls. For bafilomycin A1-treated sporozoites, in both glucose rich HBSS and HBSS without glucose, we observed no inhibition of motility (Figure 9B and 9D). By contrast, with concanamycin A treatment, there was only significant inhibition of motility at 100 µM, regardless of glucose concentration (Figure 9C and 9E).
Figure 9. Testing *P. berghei* sporozoite motility after treatment with bafilomycin A1 and concanamycin A1 in the presence or absence of an exogenous carbon source. (A) Images of *P. berghei* CSP-stained sporozoites and motility trails from control (B) bafilomycin A1-treated and (C) concanamycin A1-treated conditions. Sporozoites were preincubated in HBSS with or without glucose alone, with added with bafilomycin A1 or concanamycin A1 at either 10 µM or 100 µM, or in HBSS with cytochalasin D (1µM) for 30 minutes. Then, sporozoites were allowed to move on coverslips for 1 h in the same respective conditions. CSP on sporozoites and their shed trails were stained, and images were captured with a Nikon E600 microscope using a 40x objective lens. (D and E) Measurement and comparison of the fluorescence intensities of CSP-stained sporozoites and trails in each (D) bafilomycin A1 or (E) concanamycin A1 treatment group. Each dot represents the fluorescence intensity of one image, and 150 images were taken for each treatment group (50 per replicate well). The fluorescence intensity of each condition was compared to each other (****, *P* < 0.0001 [by a Kruskal-Wallis test followed by Dunn’s test]). Red bars represent the mean of each group.

Fatty acid-free BSA does not support sporozoite motility

Next, we sought to investigate whether fatty acids are required to sustain sporozoite motility. Although HBSS does not contain fatty acids, added BSA is required for motility, and BSA carries fatty acids on its fatty acid binding domains. We hypothesized that BSA-bound fatty acids are important for maintaining sporozoite motility. To investigate, *P. berghei* and *P. falciparum* sporozoites were preincubated for 30 min and allowed to move for 1h in HBSS with either fatty-acid free or fatty acid containing BSA at 1% (wt/vol). Additionally, sporozoites were incubated with cytochalasin D (1 µM) with or without BSA as negative controls. Multiple fatty-acid free BSA sources were tested on *P. berghei* and *P. falciparum* sporozoites to see whether BSA produced with different purification methods have varying effects on motility. When compared to motility with fatty acid free BSA, *P. berghei* sporozoites exhibited reduced circular motility in (Figure 10A). Similar reductions in motility were observed with *P. falciparum* sporozoites in the presence of several fatty acid-free BSA sources (Figure 10B). Lastly,
sporozoite motility in the presence of an additional fatty acid free BSA (A3803) was also significantly inhibited compared to motility in BSA containing fatty acids (Figure 10C). Overall, several sources of fatty-acid free BSA do not support sporozoite motility, indicating that fatty-acids bound to BSA are important for either initiating or sustaining motility.

**Figure 10.** Testing *P. berghei* and *P. falciparum* sporozoite motility in the presence of fatty-acid free BSA (A) Percentage of circular gliding *P. berghei* sporozoites in the presence of different preparations of fatty-acid free BSA, categorized/underlined by their method of purification. (B) Measurement and comparison of the area covered by CSP-stained *P. falciparum* sporozoites and trails in the presence of different fatty-acid free BSA. (C) Measurement and comparison of the fluorescence intensity of CSP-stained *P. berghei* sporozoites and trails in the presence fatty-
Discussion

The aim of this study was to characterize the role of different metabolic pathways in providing the energy that fuels sporozoite motility. Pathways such as glycolysis and oxidative phosphorylation depend on glucose, which malaria parasites can scavenge from the extracellular environment\textsuperscript{71,72}. As such, we first investigated whether sporozoite motility is dependent on exogenous carbon sources. To do this, we compared \textit{P. falciparum} and \textit{P. berghei} sporozoite motility in media containing different potential energy sources. Specifically, DMEM and RPMI contained several amino acids, pyruvate, and glucose, HBSS contained only glucose, and our lab made HBSS was created without glucose. Remarkably, we found that motility was unaffected by the exclusion of these each of these carbon sources from the media (Figure 2). Specifically, sporozoites in HBSS without glucose (which also does not contain amino acids and fatty acids) moved just as well as those in HBSS with glucose, DMEM and RPMI (Figure 2). Not only were sporozoites able move in glucose-deficient conditions, but they were able to survive and do so after a period of dissection (30-60 min) and preincubation (30 min) within the same amino acid and glucose-free media. These results indicate that sporozoites are not dependent on exogenous glucose or amino acids to sustain gliding motility. Instead, this suggests that...
endogenous energy sources, and/or in combination with exogenous energy sources scavenged while sporozoites are still in the mosquito salivary gland are sufficient for fueling motility.

Although the HBSS we tested contains no glucose, BSA is still required to initiate motility, and was added to each treatment group at 1% (wt/vol). In humans, albumin functions as a carrier molecule of several ligands including bilirubin, fatty acids, hormones, and ions. Because of its role as a carrier molecule, we hypothesized that if there were contaminating carbohydrates or amino acids carried by BSA, they could act as an exogenous energy source to induce motility in glucose-free media. Therefore, we dialyzed BSA to remove small molecules and tested motility in glucose-free HBSS. Our observation that sporozoites can move in the absence of exogenous carbon sources did not change; there was no significant difference between motility in media with dialyzed BSA and non-dialyzed BSA (Figure 3). This finding indicates that BSA contamination with small molecules such as carbohydrates or amino acids is likely not the fuel for sporozoite motility in glucose-free HBSS. However, it remains unclear whether dialysis is enough to remove tightly bound molecules from BSA. Although BSA is not generally known as a carrier of carbohydrates, it possesses binding sites for fatty acids. We later found that fatty acids bound to BSA are important for optimal sporozoite motility, where sporozoites show reduced motility in the presence of lyophilized BSA (Figure 10). Therefore, since sporozoite motility was active with dialyzed BSA, dialysis was likely not enough to remove those bound fatty acids.

Furthermore, after seeing that motility is unaffected by the absence of exogenous glucose and amino acids, we hypothesized that sporozoites could be fueling motility by extracting maximal ATP from any existing endogenous carbon sources. As such, we suspected
that they could rely on oxidative phosphorylation for energy production, a process which produces significantly more ATP than glycolysis through the breakdown of glucose and amino acids. To investigate, we treated *P. falciparum* and *P. berghei* sporozoites with atovaquone and proguanil to competitively inhibit ubiquinone and observe motility when mitochondrial electron transport is shut down. Atovaquone and proguanil are used for malaria prophylaxis, and their IC\textsubscript{50} from *in vitro* experiments against asexual erythrocytic *P. falciparum* parasites ranges between as little as 0.7-6 nM for Atovaquone and 3.2-50 µM for proguanil\textsuperscript{41}. Motility in the presence of concentrations up to 100 µM of atovaquone and proguanil did not affect motility in glucose-rich HBSS (Figure 4). By contrast, in HBSS without glucose, motility became completely inhibited for both species with as low as 1 µM atovaquone for *P. berghei* and 10 µM atovaquone for *P. falciparum* (Figure 4). These results indicate that energy output via oxidative phosphorylation is not required for motility within a glucose-rich environment. Indeed, only 1 µM of glucose was enough to overcome atovaquone+proguanil-mediated inhibition of oxidative phosphorylation (Figure 5). Thus, exogenous glucose may provide enough energy to sustain motility through glycolysis alone. However, in environments scarce of external carbon sources, oxidative phosphorylation seems to be required to sustain motility.

Next, we tested whether motility is affected by 2-DG, a glucose analog that competitively inhibits glycolysis. Like D-glucose, 2-DG is actively transported into the cell by glucose transporters and phosphorylated by hexokinase into 2-DG-6-P which directly inhibits phosphoglucoisomerase (PGI)\textsuperscript{73}. PGI cannot metabolize 2-DG-6-P; intracellular accumulation of 2-DG-6-P can exert negative feedback on hexokinase via allosteric and competitive inhibition\textsuperscript{73}. The efficacy of 2-DG has been demonstrated through use against various diseases including
cancer and viral infections, with recent approval for use in India to manage COVID-19 infection\textsuperscript{74}. Its efficacy is contingent on the dependence of cancer and virus-infected cells on glycolysis to support their high levels of anabolism, similar to blood-stage \textit{Plasmodium} parasites\textsuperscript{75}. After seeing that sporozoites can sustain motility when oxidative phosphorylation is inhibited in the presence of exogenous glucose, we hypothesized that they rely on glycolysis for ATP production. In HBSS, which contains 5.56 mM of glucose, motility was not inhibited at 2-DG concentrations up to 10 mM in \textit{P. berghei}, and up to 1 mM for \textit{P. falciparum} (Figure 6). At 10 mM, \textit{P. falciparum} motility was significantly inhibited, yet not completely (Figure 6). By contrast, in HBSS without glucose, \textit{P. falciparum} motility was completely inhibited by 100 µM, 1 mM, and 10 mM of 2-DG, while \textit{P. berghei} was partially inhibited by 100 µM and completely by 1 mM and 10 mM (Figure 6). The lack of motility inhibition in conditions with glucose is likely due to glucose outcompeting 2-DG. However, in HBSS without glucose, where there is no glucose to outcompete 2-DG, movement is inhibited. Taken together, these results suggest that sporozoites are also likely using glycolysis to sustain motility.

A caveat to these results is that 2-DG may inhibit other biological processes that depend on glucose, and those pathways may have contributed to our observed reduction of sporozoite motility. The pentose phosphate pathway (PPP) is one of those pathways. Through the PPP, glucose phosphorylated by hexokinase into G-6-P begins a series of oxidative reactions to produce ribose-5-phosphate and NADPH\textsuperscript{76}. NADPH is used by cells to control oxidative stress, while riboses are required for nucleic acid synthesis. In \textit{P. falciparum} blood stage parasites, glucose flux through the PPP is increased to drive nucleic acid synthesis and reduce oxidative stress during proliferation\textsuperscript{26}. While sporozoites do not have the same anabolic demand for
nucleic acids as proliferating erythrocytic parasites, they still express enzymes of the PPP\textsuperscript{42}, and NADPH-dependent control of oxidative stress may be important for maintaining motility. However, it is thought that 2-DG does not inhibit the PPP unless used at high concentrations above 20 mM, where a build up of 2-DG-6-P can allosterically and competitively inhibit hexokinase to block the production of G-6-P\textsuperscript{77}. By contrast, at lower concentrations, PGI inhibition by 2-DG could cause an accumulation of unused G-6-P in the cell that can shunted into the PPP, and therefore, may increase PPP flux as opposed to inhibiting it\textsuperscript{77}. Because we observed inhibition of motility with as low as 100 µM in \textit{P. falciparum}, where hexokinase activity would still be active, we speculate that it is unlikely that PPP inhibition contributed to motility inhibition. Furthermore, these assays were conducted over a short time frame, 1 to 1.5 hours, and it is unlikely that any changes in nucleic acid production or redox flux are unlikely to have had an impact in this time frame.

Apart from PPP interference, 2-DG also inhibits mannose dependent pathways. This is because D-glucose and D-mannose are epimers only differing in the planar position of the hydroxyl group bonded to their second carbon. As such, removal of the hydroxyl group in mannose produces 2-DG’s molecular structure—the compound is also known as 2-deoxy-D-mannose. Because of its likeness to mannose, 0.5 mM of 2-DG has been shown to interfere with protein glycosylation in cancer cells\textsuperscript{78,79}. In \textit{Plasmodium}, two important mannose-dependent glycosylation processes are the GPI-anchoring of proteins and the C-mannosylation of thrombospondin type I repeat (TSR) domains\textsuperscript{80,81}. GPI-anchored proteins such as CSP are essential for sporozoite development and motility\textsuperscript{82}. Because CSP is shed during motility, GPI may need to be constantly synthesized to replace shed CSP on the parasite surface. In addition,
C-mannosylation is important for sporozoite motility because the TSR domain of TRAP is C-mannosylated.\(^{80}\) Thus, off-target inhibition of the C-mannosylation catalyzing enzyme (DPY19) with 2-DG has potential to hinder gliding.\(^{83}\) Indeed, *P. berghei* DPY19 knockout ookinetes, where the wildtype DPY19 gene was replaced by a knockout cassette, show an inability to glide.\(^{84}\) As such, whether 2-DG interferes with motility by interfering with GPI anchoring and/or C-mannosylation requires further investigation. This could be done by metabolic labeling experiments to look at CSP and TRAP synthesis in the presence of 2-DG.

Aside from glucose-based metabolism, we investigated whether PbVP2, encoding a PP\(_i\) hydrolyzing V-type H\(^+\)-PPase, is important for sporozoite motility. *P. berghei* sporozoite motility was not significantly inhibited in the presence of PP\(_i\) analogues AMDP and pamidronate, and the PP\(_i\) hydrolysis inhibitor IDP in both the presence and absence of exogenous glucose (Figure 7). These results indicate that the function of PbVP2 is not important for sustaining motility. However, it is possible that the loss of proton pumping from PbVP2 can be compensated by the function of other V-ATPases which couple ATP hydrolysis to pump protons. Since we found that sporozoite motility is not inhibited in the absence of glucose, glucose-free HBSS is perhaps not sufficient to mimic ATP scarce conditions that would also inhibit ATP hydrolysis by V-ATPases. Furthermore, against *T. gondii* tachyzoites, 100 µM AMDP and 75 µM pamidronate inhibited 25% and 75% of tachyzoite proliferation within human foreskin fibroblasts respectively.\(^{85}\) AMDP and pamidronate were also shown to inhibit PPI-dependent proton pumping respectively by 80% and 50% at as low as 2 µM and 20 µM.\(^{85}\) Similarly, 25 µM IDP was shown to inhibit PPI hydrolysis by 60% in tachyzoite homogenates.\(^{85}\) However, against *Plasmodium* sporozoites, it remains unclear whether these compounds can successfully penetrate the cell and act on their
intended pathway. Even if the compounds enter the cell, analogues of PP\(_i\) may have off target effects. Because PP\(_i\) is a by-product of reactions that involve ATP hydrolysis, the accumulation of PP\(_i\) can exert negative feedback on biological pathways that produce it; PP\(_i\) analogues may mimic this negative feedback. To further understand the importance of PP\(_i\) and specifically V-type H\(^+\)-PPases in sporozoites we are in the process of investigating PbVP2 knockout parasites.

Additionally, we investigated how the inhibition of V-ATPases with selective inhibitors bafilomycin A1 and concanamycin A affects \(P.\) \textit{berghei} sporozoite motility. Motility was only significantly inhibited when incubated with 100 µM of concanamycin A in both HBSS with and without glucose (Figure 9). No inhibition was observed when treated with bafilomycin A1 (Figure 9). Both concanamycin A and bafilomycin A1 have been shown to inhibit \(P.\) \textit{falciparum} growth \textit{in vitro} at low micromolar-nanomolar concentrations, and 1 µM of bafilomycin A1 was sufficient to hinder sporozoite vacuolar egress during invasion of HepG2 cells\(^{62,86}\). With activity at these low concentrations, we expected 10 µM and 100 µM to be more than sufficient to inhibit V-ATPase activity. Having observed no inhibition with bafilomycin A1 at up to 100 µM, and concanamycin A at 10 µM, sporozoite motility is likely not dependent on V-ATPase activity. Because bafilomycin A1 and concanamycin A both selectively inhibit V-ATPases, any observed inhibition of motility was expected to be observed after treatment with both inhibitors. As such, inhibition with 100 µM of concanamycin A alone may have been caused by off-target effects or toxicity at a high concentration. However, the motility of sporozoites incubated with 100 µM of concanamycin was not completely inhibited; they left behind fewer trails that were irregular and more linear (Figure, 9C) indicating that they were not killed during preincubation. It is possible that the loss of proton-pumping by V-ATPases can be compensated by other
proton pumps such as VP2. Overall, it appears that targeting V-ATPases alone is not sufficient to inhibit sporozoite motility.

Lastly, our study of motility in the presence of fatty acid-free BSA indicated that fatty acids play an essential role in inducing motility. For *P. berghei*, *P. falciparum*, and *P. yoelii* sporozoites, motility was significantly reduced when incubated with multiple sources of fatty acid-free BSA opposed to BSA purified in a way that maintained its fatty acid content (Figure 8). The mechanism by which fatty acids are stimulating motility remains unclear. It is unlikely that sporozoites are metabolizing down fatty acids to produce energy since malaria parasites lack enzymes required for β-oxidation. However, sporozoites do have the capability to perform fatty acid synthesis through the FAS-II pathway\textsuperscript{66,67,87}. Synthesis occurs through stepwise elongation of a fatty acid chain with 2 carbons added onto the chain per cycle\textsuperscript{87}. Fatty acids produced by the FAS-II pathway can be further elongated on the cytoplasmic surface of the ER through the elongase (ELO) pathway\textsuperscript{88}. The ELO pathway uses acetyl-CoA as opposed to acyl carrier protein in the FAS-II pathway, and therefore requires separate enzymes to grow the fatty acid chain\textsuperscript{89}. Since these synthetic pathways exist, it puts into question whether they can operate in reverse to break down fatty acids from BSA as an alternative to β-oxidation. The FAS-II pathway is carried out by four main enzymes encoded by the genes: *fabB/F*, *fabG*, *fabI*, and *fabZ*\textsuperscript{87}. Deletion of *fabB/F* and *fabI* have been reported to abrogate *P. falciparum* sporozoite development in the mosquito midgut\textsuperscript{66}. However, this phenotype was not observed with *P. berghei* and *P. yoelii*, where knockout of the same enzymes only hindered liver stage progression\textsuperscript{67}. This interspecies difference is likely because *P. falciparum* produces larger oocysts than *P. berghei* and *P. yoelii* (S. Kanatani, unpublished data). Because knockout of FAS-II
enzymes does not hinder *P. berghei* and *P. yoelii* parasites prior to the liver stage, it is likely not important for sporozoite motility. Instead, we speculate that the inhibition of motility observed in fatty-acid-free BSA is likely caused by a separate pathway.

Another way in which fatty acids may stimulate motility is through signaling. It is thought that albumin induces sporozoite motility by interacting with the cell surface and initiating a signal transduction cascade\(^44\). Because albumin binds fatty acids, it is possible that when lyophilized, BSA loses the tertiary structure required for cell-surface interaction and induction of signaling. Therefore, fatty acids may not be required as an energy source for motility, but instead as a component of BSA which is necessary for a signalling pathway required for the sporozoite to switch from a non-motile to a motile state once it’s inoculated into the mammalian host. Altogether, BSA-bound fatty acids play an important part in stimulating motility, yet further experiments are required to understand their role.

A limitation of these experiments is that they only capture the initial 15 minutes of sporozoite motility. For fully motile untreated sporozoites, after 15 minutes, no additional fluorescence intensity was detected at longer time points up to 1 h (Supplementary Figure 2). Sporozoites in HBSS without glucose achieved their peak intensity as fast as those in HBSS with glucose (Supplementary Figure 2). Although a fraction of sporozoites may only require a few minutes to successfully enter the bloodstream from the dermis, the majority take 1 to 2 hours. In *vivo* sporozoites can move for over 2 hours\(^90\). In the future, it would be interesting to investigate whether treatment with inhibitors/conditions that did not show motility inhibition using this fluorescence assay (AMDP, pamidronate, IDP, bafilomycin A1, and HBSS without glucose) have observable effects on the duration of motility.
Altogether, our finding that both atovaquone+proguanil and 2-DG inhibit motility in HBSS without glucose indicates that motility can be driven by both glycolysis and oxidative phosphorylation. Remarkably, motility is unperturbed in the absence of exogenous carbohydrates and amino acids. This finding prompted our investigation of alternative energy sources, where we found that inhibition of H⁺-PPases with PPi analogues and hydrolysis inhibitors had no effect on motility. Investigation of a PbVP2 knockout line is ongoing. Additionally, we conclude that inhibiting V-ATPases alone is not sufficient to affect motility. Lastly, our findings indicated that fatty acids bound to albumin are important for promoting motility. Identifying these metabolic pathways that mediate sporozoite motility may help reveal new targets for the development of transmission-blocking compounds.
References


Supplementary Materials

Supplementary Figure 1. The fluorescence intensity of images taken for our motility assays were measured on ImageJ with the settings and code shown above using the macro batch processing option on ImageJ.

Supplementary Figure 2. Differences in *P. berghei* sporozoite motility in HBSS with or without glucose overtime. Sporozoites were preincubated for 30 min in HBSS with or without glucose,
or in HBSS with cytochalasin D (1µM). Then, sporozoites were allowed to move on coverslips for either 5, 15, 45, or 60 minutes. Measurement and comparison of the fluorescence intensities of CSP-stained sporozoites and trails in each treatment group was conducted. Each dot represents the fluorescence intensity of one image, and 50 images were taken for each treatment group (25 per replicate well). The fluorescence intensity of each condition was compared to each other (****, \(P < 0.0001\) [by a Kruskal-Wallis test followed by Dunn’s test]). Red bars represent the mean of each group. Here, both HBSS with or without glucose are shown to move and leave behind enough CSP-stained trails to produce their peak fluorescence within the first 15 minutes.

**Supplementary Table 1: Primers Used for PbVP2 Transfection**

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<th>Primer Name</th>
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<tr>
<td>2</td>
<td>GT</td>
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</tr>
<tr>
<td>3</td>
<td>QCR1</td>
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<td>4</td>
<td>QCR2</td>
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<td></td>
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**Supplementary Table 2: Formulation of Lab-made HBSS without Glucose**

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<td>Calcium Chloride (CaCl(_2)) (anhyd.)</td>
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<td>Magnesium Chloride (MgCl(_2)-6H(_2)O)</td>
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<td>Magnesium Sulfate (MgSO(_4)-7H(_2)O)</td>
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<td>Sodium Chloride (NaCl)</td>
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