RUNNING TITLE: Sporozoite longevity buffer

TITLE: Enhancing longevity of *Plasmodium vivax* & *P. falciparum* sporozoites after dissection from mosquito salivary glands

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Abbreviations:

Grace's (Grace's Insect Medium)
RPMI (RPMI 1640 Medium)
HBSS (Hank's Balanced Salt Solutions)
PBS (Phosphate-Buffered Saline)
MGP (mean gliding percentage)

ABSTRACT

The pre-erythrocytic stages of *Plasmodium vivax* and *P. falciparum* remain challenging for experimental research in part due to limited access of sporozoites. An important factor limiting availability is the laboratory support required for producing infected mosquitoes and the ephemeral nature of isolated extracellular sporozoites. This study was undertaken to investigate methods to improve availability of this limited resource by extending the longevity of the extracellular sporozoites after mosquito dissection. Our goal in this study was to determine whether buffer conditions more closely mimicking the insect microenvironment could prolong longevity of *ex vivo* *P. vivax* and *P. falciparum* sporozoites. The study compared the current standard dissection buffer RPMI1640 to Hank's Balanced Salt Solution with 1g/L glucose (HBSS-1) or 2g/L glucose (HBSS-2) and Grace's Insect Medium for ability to extend longevity of *ex vivo* *P. vivax* and *P. falciparum* sporozoites. The effect of each buffer on sporozoite viability was evaluated by measuring sporozoite gliding motility at 0, 4, 8, and 24 hours post-dissection from mosquito salivary glands. Comparisons of mean gliding percentages of *ex vivo* sporozoites in the different buffers and time points found that RPMI and Grace's both showed strong gliding at hour 0. In contrast, by 4 hours post-dissection sporozoites in RPMI consistently had the lowest gliding activity, whereas sporozoites in Grace's had significantly more gliding compared to all other buffers at almost all time points. Our results indicate that *P. vivax* and *P. falciparum* sporozoites maintained in insect media rather than the standard dissection buffer RPMI and HBSS retain viability better over time.

1. Introduction

1.1. Background

The World Health Organization estimated that malaria caused 207 million cases in 2012, with 627,000 resulting deaths [1]. In order to reduce this burden, research is being conducted in the fields of drug discovery and vaccine development, with a particular emphasis on the pre-erythrocytic stages of the malaria life cycle. Discovery of new drugs as well as development of effective vaccines is extremely important due to the emergence of resistance to antimalarial drugs and insecticides to control mosquito transmission. Efforts to contain emerging antimalarial drug resistance are limited by the lack of effective preventative treatments for radical cure of liver-stage parasites except for 8-aminoquinolones. In particular, to prevent relapsing malaria caused by *Plasmodium vivax* and *P. ovale*, treatment regimens targeting dormant liver-stage parasites (hypnozoites) are needed [2].

Studying the liver stage of malaria parasites requires access to sporozoites, the hepatocyte infecting life cycle stage of the parasite transmitted by mosquito bite. In laboratory settings sporozoites are typically produced through artificial membrane feeding technology that allows mosquitoes to be infected with *P. falciparum* and *P. vivax* without the need to take an infected blood meal directly from a malaria patient. For *P. falciparum*, infectious blood can be generated from *in vitro* gametocyte cultures derived from continuous asexual culture of numerous culture-adapted lines and clinical isolates. In contrast, continuous *in vitro* culture methods have not been established for *P. vivax* [3]. Thus *P. vivax* gametocyte-infected blood must come from clinical isolates or non-human primate models [4, 5]. After 14-20 days post infective blood meal, sporozoites of *P. falciparum* and *P. vivax* can be harvested from salivary glands of infected mosquitoes, the organ of the female mosquitoes in which the infectious sporozoites reside. However, these procedures require specialized insectary facilities that have adequate biosafety requirements to contain the infected mosquitoes. While infectious sporozoites are present in the mosquito for 8-21 days, they remain viable for a relatively short time once dissected out of the salivary glands [6]. These factors and other resource limitations restrict access to sporozoites for liver-stage study of *P. falciparum* and *P. vivax*.

Historically, many different medias and additives have been utilized for salivary gland dissections such as TC Medium 199, bovine serum albumin (BSA), Alsever solution with glycerol, Whitman's fluid, golden hamster plasma, dimethylsulfoxine, and inositol [7]. Saline, heparinized chicken plasma, chicken erythrocyte extract, and skim milk have also been used [8-10]. More recently media including RPMI, HBSS, Leibovitz, and Grace's have been employed for salivary gland collection [11-13]. Various studies have examined the effects of buffers and other *in vitro* conditions on sporozoites for various *Plasmodium* species in efforts to extend their longevity after isolation from mosquito salivary glands [8-10, 14-16]. More recently, a contrasting approach evaluated conditions that might stimulate sporozoite transformational development as would happen in the mammalian host to develop an automated drug assay screen for inhibitors of *P. berghei* salivary gland sporozoite development [14]. Results of the *P. berghei* study found that sodium bicarbonate was a critical component that initiated

sporozoite transformation, serum albumin prolonged viability, and different amounts of glucose affected transformation and death differently depending on the buffer used [14]. The prolonged viability of *P. berghei* sporozoites as a result of serum albumin was demonstrated by increased half-life of sporozoites by four hours in RPMI and greater than 9 hours in HBSS [14]. Additionally, serum albumin extended the amount of time post dissection before sporozoite transformation by two hours in RPMI and by greater than 9 hours in HBSS [14]. The enhancing effects of serum albumin over time had previously been established in *P. berghei* sporozoites through a motility study in which serum albumin not only activated sporozoite motility but also prolonged that activity with little to no attenuation for up to 24 hours [17].

In order to increase sporozoite access, improved methods are needed to preserve sporozoite viability *ex vivo*. One approach to extend viability is to optimize the buffer used to collect sporozoites after dissection, potentially allowing for transport to other laboratories without specialized insectary facilities. Currently, the mammalian cell culture medium RPMI 1640 (RPMI) is used to isolate sporozoites *ex vivo* since it is compatible to the cell culture medium used for maintenance of hepatocyte cultures and is compatible for intravenous or intraperitoneal injection into laboratory animals. However, RPMI has limited preservation qualities for sporozoites [14], requiring groups that do not have direct sporozoite access within their own laboratory facilities to travel to perform experiments at adequately equipped collaborating laboratories. For groups studying *P. vivax*, this usually requires international travel and extensive IRB approval to work with field isolates. Current solutions to these access problems include shipping infected blood (cryopreserved or fresh) and shipping infected mosquitoes. In addition to the logistical and regulatory challenges, viability of parasites is decreased through these methods. Improved methods to extend sporozoite viability over time may be able to increase laboratory access to sporozoites through commercial courier services to build additional capacity for essential *P. falciparum* and *P. vivax* liver-stage research.

1.2. Objectives

The goal of this study was to evaluate the standard dissection medium RPMI against different modifications of HBSS, a buffered salt solution used for mammalian cells, and Grace's Insect Medium (Grace's), an insect buffered medium, to determine if an alternate dissection buffer could improve longevity of sporozoite viability. In particular, we hypothesized that using a dissection buffer that better mimics the inorganic salts of the mosquito salivary gland environment may further extend viability of dissected sporozoites. Sodium bicarbonate was minimized in the buffers tested since it activates sporozoite transformation and albumin was included in all buffers evaluated since it extends sporozoite viability in the absence of bicarbonate. For the current studies we evaluated these variables for their effect on longevity of *ex vivo* *P. falciparum* and *P. vivax* sporozoites. Sporozoite gliding motility was used as an indicator of viability, since it is an essential function for migration and cell traversal by which sporozoites move to infect liver cells using membrane-associated actin myosin motors and the *in vitro* gliding assay represents a simple yet efficient quantitative tool to assess sporozoite viability [17-20].

2. Materials and Methods

2.1. Ethics Statement

The Human Subjects protocol for this study was approved by the Ethical Review Committee of Faculty of Tropical Medicine, Mahidol University (MUTM 2011-040-03). All subjects or their legal guardians provided written informed consent to participate in the study.

2.2. *P. vivax* mosquito infections from infected patient blood

Isolates of *P. vivax* were collected from symptomatic patients enrolled in the study after giving written informed consent at Thai malaria clinics in Kanchanaburi province (IRB approved protocol number TMEC 11-033). A sample of *P. vivax*-infected blood was drawn by venipuncture into heparin tubes. Thick and thin Giemsa-stained smears were prepared after removal of white blood cells. A diagnostic PCR was used to confirm microscopic identification that *P. vivax* was the only *Plasmodium* species present in the samples used for the study [21].

The mosquito infections for *P. vivax* were performed using a colony of *Anopheles dirus* (Bangkok strain). Briefly, 150 µl of RBC pellet from infected patient blood samples was suspended in pooled normal AB serum to 50% hematocrit. Next, 300 µl of the suspension was fed for 30 minutes to 5–7 days old female mosquitoes via an artificial membrane attached to a water-jacketed glass feeder maintained at 37°C. Engorged mosquitoes were kept on 10% sucrose solution and maintained at 26°C and 80% humidity until dissected.

2.3. *P. falciparum* infections from in vitro cultured parasites

A cloned line of *P. falciparum* NF54 was maintained according to standard methods at 37°C (5% O₂ and 5% CO₂, nitrogen balanced) in 6% hematocrit (O+ blood) and RPMI 1640 medium with 10% AB human serum and 2.5% sodium bicarbonate with no antibiotics [22]. Two gametocyte cultures were started one day apart using standard methods and an aliquot of each was mixed together when stage V gametocytes were prevalent (day 14-17 of gametocyte cultures) and used to infect mosquitoes. Infections of laboratory-reared *An. stephensi* (Nijmegen strain) were accomplished using a temperature-controlled Hemotek membrane-feeding device (Discovery Workshops).

2.4. Experimental Design

A total of eight experiments were performed for this study, six on *P. vivax* and two on *P. falciparum*. Experimental group *P. vivax*-A consists of three experiments conducted in Thailand, and experimental group *P. vivax*-B consists of three experiments conducted in the United States using mosquitoes shipped to the US after being reared and infected in the same Thai setting as group *P. vivax*-A. Tested buffers were RPMI (Life Technologies, cat. no.31800-089), two variations of HBSS (Life Technologies, cat. no.14175-095), one variation of Grace's (Life Technologies, cat. no.11300-027), and

phosphate-buffered saline (PBS) (Life Technologies, cat. no.18912-014) used as a control. Infected mosquitoes were dissected for salivary glands to isolate sporozoites. Salivary glands were directly dissected into test buffers on ice or dissected into PBS on ice and distributed into test buffers post-dissection. Salivary glands were ruptured immediately after dissection for quantification of sporozoites in order to appropriately dilute and aliquot parasites. Gliding assays were initiated with sporozoites from each buffer at 0, 4, 8, and 24 hours post-dissection. Sporozoites and buffers remained at room temperature from completion of dissection until used in gliding assays.

2.2. Buffer Preparation

All buffers were prepared using distilled water. BSA was added to all test buffers at 30 mg/L and all test buffers were pH-balanced to 7.0-7.4. Glucose was added when necessary to achieve final glucose concentrations of 2 g/L in RPMI, 1 g/L in HBSS-1, 4 g/L in HBSS-2, and 0.7 g/L in Grace's. All buffers were filter-sterilized prior to use. Buffers contained minimal or no sodium bicarbonate (Table A.1).

2.3. Sporozoite Gliding Motility Assay

The gliding motility assay protocol was modified from previous studies [23]. Briefly, for each assay 30,000 sporozoites in one of the test buffers were incubated in a 24-well plate at 37°C for 30 minutes on a glass cover slip coated with anti-circumsporozoite protein (CSP) (monoclonal antibody (MAB) 2A-10 (MRA-183) for *P. falciparum* and for MAB 2F2 (MRA-184) *P. vivax* VK210) [24, 25]. Coverslips were immersed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature to fix attached sporozoites and protein gliding trails, followed by treatment with a blocking buffer (1% BSA in PBS) for 15 minutes at room temperature. Sporozoites and gliding trails were subsequently visualized by IFA with anti-CSP primary antibody and secondary anti-mouse Alexa Fluor 488. Coverslips were mounted on glass slides upside down using Fluoromount G and stored at 4°C until analyzed by fluorescence microscopy.

2.4. Determination of Sporozoite Gliding

Sporozoites and their trails were viewed using an inverted fluorescent Zeiss Axiovision microscope at 40X. Each assay was quantified by averaging the gliding percentages from 20 random fields of vision. Gliding percentages for each field were calculated by dividing the total number of gliding sporozoites with trails by the total number of sporozoites within the field of vision. Sporozoites were considered to be gliding if they had an attached trail at least the length of the actual sporozoite.

2.5. Statistical Analysis

Statistical analysis was performed using SAS software (version 9.1.3; SAS Institute, Cary, NC). Gliding percentage distributions for each buffer and time point per experimental group (*P. vivax*-A, *P. vivax*-B, and *P. falciparum*) were assessed for

normality using the Shapiro-Wilk test for normality in combination with box plots and normal probability plots. A repeated measures ANOVA was performed to test the statistical significance of the influence different factors had on gliding percentage. The within-experiment factors tested were “buffer” (RPMI, HBSS-1, HBSS-2, Grace’s, PBS) and “time” (0, 4, 8, 24 hours), while the between-experiment factor tested was “experimental group” (*P. vivax*-A, *P. vivax*-B, *P. falciparum*). All further analysis was conducted with the data stratified by experimental group.

For normally distributed gliding percentages, two-tailed Student’s t-tests were used to determine if the mean gliding percentage (MGP) for each buffer within each time point and experimental group was statistically different from 0. For non-normal distributions, the sign test was used in place of Student’s t-test. For normally distributed gliding percentages, two-tailed paired t-tests were conducted to compare the MGP for each buffer to the MGP for RPMI and for Grace’s within each time point and experimental group. For non-normal gliding percentage distributions, the Wilcoxon signed rank test was used in place of the paired t-test. To control family-wise error rates due to multiple comparisons, a Bonferroni adjustment was applied to the level of significance for the comparison tests.

3. Results

3.1. Temporal Trends in Sporozoite Viability

Sporozoite viability observed by the standard gliding assay generally trended down over time for each species of parasite, each buffer, and each experimental group (Fig. 1). Both the number of sporozoites capable of gliding and the robustness or extent of their gliding activity appeared to diminish over time (Fig. 1). To determine if differences between buffers and time points were significant, the mean gliding percentages (MGP) were determined for each experimental group (Table 2). These tabulated data were converted into graphs to facilitate comparisons (Fig. 2).

The variables of time and buffer had independent and dependent significant influences on sporozoite viability evident in differences of gliding percentage (Table 1: ANOVA Time $p < 0.0001$, Buffer, $p < 0.0001$, Time*Buffer $p < 0.0001$). Additionally, the influences of time and buffer on gliding percentage were both statistically dependent on the experimental group (Table 1: Time*Group $p < 0.0001$, Buffer*Group $p < 0.0001$). The dependent influence of time and buffer on gliding percentage was also statistically dependent on experimental group (Table 1: Time*Buffer*Group $p < 0.0001$).

3.2. Detailed analysis provides insight into the basis of significant influences on sporozoite viability.

In experimental group *P. vivax*-A, test buffers had an initial MGP between 43.93-58.28% and final average percentages between 3.46-22.66% (Table 2). As seen in Fig. 2.A, RPMI had one of the highest initial MGPs but had the lowest MGP of all test buffers by 4 hours (Fig. 2.A). RPMI remained the buffer with the lowest MGP through 8 hours and 24 hours (Fig. 2.A). In comparison, Grace’s had the lowest initial MGP of all the test buffers, but increased in MGP through 4 and 8 hours (Fig. 2.A). This increase made

Grace's the buffer with the highest MGP at 4 and 8 hours (Fig. 2.A). By 24 hours, Grace's still had one of the highest MGPs (Fig. 2.A). HBSS-1 and HBSS-2 had comparable initial MGPs to each other and RPMI, and decreased over time similarly to RPMI (Fig. 2.A). However, both HBSS buffers maintained higher MGPs than RPMI at 4, 8, and 24 hours (Fig. 2.A). HBSS-2 had a lower initial MGP compared to HBSS-1, but decreased at a slower rate (Fig. 2.A). HBSS-2 was comparable to Grace's at 24 hours with one of the highest MGPs (Fig. 2.A).

In experimental group *P. vivax*-B, test buffers had an initial average MGP between 11.19-32.65% and final average percentages between 0-5.24% (Table 2). As seen in Fig. 2.B, RPMI had one of the highest initial MGPs but essentially had no gliding by 4 hours (Fig. 2.B). RPMI was statistically or otherwise equivalent to 0 by 8 and 24 hours (Table 2). In comparison, Grace's also had one of the highest initial MGPs (Fig. 2.B). Grace's gliding dropped significantly by 4 hours, but the decrease was not as great as RPMI's decrease (Fig. 2.B). Grace's maintained the highest MGP at 4, 8, and 24 hours (Fig. 2.B). HBSS-1 and HBSS-2 had significantly lower initial MGPs than RPMI and Grace's (Fig. 2.B). HBSS-2 had a higher initial MGP compared to HBSS-1, and decreased at a slower rate (Fig. 2.B). HBSS-2 had the second highest MGP at 4, 8, and 24 hours (Fig. 2.B).

In *P. falciparum*, test buffers had initial MGPs between 31.89-56.52% and final average percentages between 0-6.73% (Table 2). As seen in Fig. 2.C, RPMI only had the third highest initial MGP (Fig. 2.C). RPMI MGP was statistically equivalent to 0 by 4 hours, and equaled 0% by 8 and 24 hours (Table 2). In comparison, Grace's had the highest initial MGP, and remained the highest over time with a slow and steady decrease (Fig. 2.C). HBSS-1 and HBSS-2 also had significantly lower initial MGPs than Grace's (Fig. 2.C). However, HBSS-2 had higher initial average gliding than RPMI, while HBSS-1 had lower initial gliding than RPMI ((Table 2, Fig. 2.C). Both HBSS buffers decreased at a rate similar to RPMI having essentially no gliding by 8 hours and on (Fig. 2.C).

3.3. Statistical Comparisons Between Buffers

As seen in Table 2, all test buffers in group *P. vivax*-A were statistically different from 0 at all time points. At 0 hours, RPMI had the second highest MGP but was not statistically different from the highest initial MGP (Table 2). Both RPMI and HBSS-1 had statistically higher initial MGPs than Grace's (Table 2). By 4 hours, Grace's had the statistically highest MGP (Table 2). RPMI, HBSS-1, and HBSS-2 had no statistical difference at 4 hours (Table 2). By 8 hours, Grace's still had the statistically highest MGP (Table 2). HBSS-2 MGP at 8 hours is statistically higher than RPMI (Table 2). By 24 hours, Grace's and HBSS-2 had the highest MGPs but were not statistically different (Table 2). At 24 hours Grace's MGP was statistically different from RPMI (Table 2), and RPMI was statistically equivalent to the control, PBS (Table 2).

In group *P. vivax*-B, RPMI and Grace's had the two highest MGPs and were not statistically different from each other at 0 hours (Table 2). Both however, were statistically different from all other buffers (Table 2). At 4 hours, Grace's and HBSS-2 had the highest MGPs and were not statistically different from each other (Table 2). At 4 hours both Grace's and HBSS-2 were statistically different from RPMI (Table 2). By 8

hours, RPMI MGP was statistically equivalent to 0 (Table 2), and Grace's had the statistically highest MGP (Table 2). By 24 hours, Grace's still had the statistically highest MGP (Table 2). At 24 hours, Grace's and HBSS-2 were then the only two buffers statistically different from 0 (Table 2).

In group *P. falciparum*, Grace's had the statistically highest MGP at 0 hours (Table 2). Grace's still had the statistically highest MGP through 4, 8, and 24 hours (Table 2). RPMI was statistically equivalent to 0 by 4 hours, and equaled 0 at 8 and 24 hours (Table 2). At 24 hours, HBSS-1 was the only buffer other than Grace's with an MGP statistically different from 0 (Table 2).

3.4. Experimental Group Comparison

For all experimental groups, Grace's had higher MGPs than all other buffers at 4, 8, and 24 hours with only two exceptions. At 24 hours in group *P. vivax*-A and at 4 hours in group *P. vivax*-B, Grace's MGP was statistically equivalent to HBSS-2. In experimental groups *P. vivax*-B and *P. falciparum*, Grace's had the highest MGP at 0 hours as well. In experimental group *P. vivax*-A, Grace's had a relatively comparable MGP to the other test buffers at 0 hours. Unique to this experimental group, Grace's had an increase in MGP up until 8 hours. RPMI had the highest or was statistically tied for the highest initial MGP in groups *P. vivax*-A and *P. vivax*-B. RPMI had the lowest MGP of all test buffers at 4, 8, and 24 hours in all three experimental groups.

4. Discussion

Sporozoites of malaria parasites complete their development inside the microenvironment of the *Anopheles* salivary gland. Once developed, sporozoites must wait quiescently in the salivary glands for an extended period of time, until the mosquito takes another blood meal, to be injected with some saliva and infect a new vertebrate host [16, 26, 27]. In contrast, once they are removed from the salivary glands in the laboratory viability of sporozoites rapidly dissipates [28]. Similar to other parasitic protozoans transmitted by hematophagous insects *Plasmodium* sporozoites must be prepared for the major changes in their microenvironment when injected with the saliva of their vector into the skin of their vertebrate host. Therefore, the quiescent salivary gland sporozoites are probably in a primed condition, sensitive to external stimuli, which during dissection may enable activation of the sporozoites' invasion-related processes. To further understand the state of salivary gland sporozoites, several studies have investigated their motility at different stages of maturation and invasion mechanisms used in the salivary gland [17, 29]. In the motility study, oocyst sporozoites demonstrated gliding in only 5% of parasites where as 90% of salivary gland sporozoites exhibited gliding capabilities, implying that gliding is an attribute sporozoites develop as they mature [17]. Furthermore, salivary gland sporozoites were 1000-fold more infective to host hepatocyte cells than oocyst sporozoites, demonstrating a relationship between gliding motility and hepatocyte invasion [16]. Both oocyst and salivary gland sporozoites express the surface protein thrombospondin-related adhesion protein (TRAP), which has been demonstrated to be a necessary component of salivary gland invasion and hepatocyte cell invasion [29-33]. Additionally, TRAP is

necessary for gliding in both oocyst and salivary gland sporozoites [29]. While TRAP is necessary for gliding, it is not indicative of the sporozoite's ability to glide as demonstrated by the presence of TRAP on oocyst sporozoites, the majority of which don't glide. The maturation process that gives a sporozoite its ability to glide likely involves functional changes in TRAP. It is not fully understood at which point in maturation sporozoites gain their ability to glide and thus it is not known whether gliding is a necessary component of salivary gland invasion. Further, while 90% of salivary gland sporozoites show gliding capability, it is not clear whether gliding occurs within the salivary gland, or if the primed condition of sporozoites that makes it sensitive to external stimuli allows for activation of sporozoite gliding only once outside salivary glands. A number of studies indicate a calcium-dependent kinase signaling pathway is involved in activating sporozoites [11, 34-37]. BSA has been shown to prolong activation in *P. berghei* sporozoites for up to 24 hours [17]. Regardless, while activation of sporozoite gliding may not be necessary for salivary gland invasion, it is a critical component of host hepatocyte cell invasion and is thus an integral part of sporozoite viability and longevity once isolated from the mosquito [18, 39, 40].

Unfortunately, current understanding of the complex biological processes of sporozoite infection is inadequate and therefore the properties that avoid premature activation of salivary gland sporozoites remain challenging to manage during experimental laboratory studies. This is especially true for the human malaria parasites *P. falciparum* and *P. vivax*, where methodological constraints and laboratory infrastructure requirements further restrict the number of potential studies to understand these biological processes. To improve experimental use of human malaria sporozoites this study examined alternate buffers for isolation of sporozoites in salivary gland dissection that can potentially be controlled to extend viability of *P. falciparum* and *P. vivax* sporozoites for use in experimental studies. The Grace's insect medium outperformed the mammalian buffers and medium at nearly every time point for both *P. falciparum* and *P. vivax* sporozoites.

A number of studies have investigated the effects of different buffers and other variables for effect on sporozoite viability [8-10, 15, 16]. *In vitro* studies indicate that sodium bicarbonate and elevated temperature can initiate transformation, and therefore bicarbonate was minimized or not used in any of the media evaluated [14]. In contrast, serum albumin in the absence of bicarbonate has been shown to prolong viability; therefore, 30 mg/L of BSA was included in all media evaluated. Thus the fundamental evaluation in our study compared cell culture solutions used for mammalian cells, RPMI and HBSS at two different glucose concentrations, to a medium intended for use with insect cells, using PBS as a reference. A complete list of ingredients for the basic buffers used in this study can be viewed in the appendix. While the only ingredients in HBSS include inorganic salts and glucose, RPMI and Grace's both include inorganic salts, glucose, vitamins, amino acids, and other components. Vitamins and amino acids are added to culture media to aid in the proliferation and growth of cells, while inorganic salts in media creates osmotic balance. Since growth and proliferation of cells is not directly relevant to sporozoite viability outside of the salivary glands, we will limit our discussion to inorganic salts, the only category of ingredients common to all test buffers.

The main differences between mammalian cell and insect media are the pH and the inorganic salt content [41]. Insect media are typically more acidic to match the lower

pH of insect cells [42]. The osmotic pressure of insect cells can be up to double that of mammalian cells, requiring drastically different inorganic salt concentrations in the respective media [41]. While RPMI and HBSS share some overlap in inorganic salts and concentrations used in both media, Grace's has only two inorganic salts (KCl, KH_2PO_4) in common and they are used at considerably different concentrations [43]. These differences in inorganic salts and their concentrations account for the difference in osmotic pressure between mammalian and insect cells. On the other hand a *Plasmodium* sporozoite is a protozoa, neither a mammalian nor an insect cell. However, a sporozoite's physiologic adaptation to the *Anopheles* salivary glands enables them to develop and remain there for an extended period in suspended animation before infection of the vertebrate host. During the transition from this invertebrate microenvironment into the vertebrate host, sporozoites are activated by some of the extrinsic factors present. As noted there is evidence to indicate that *in vivo* activation involves signaling pathways that occur in response to a combination of environmental, chemical, and physical interactions although the sequence and priority of these complex signals and their functional mechanisms remain inadequately identified [35, 44, 45]. The migratory phase eventually brings sporozoites into the liver where cell traversal occurs and eventually sporozoites enter a hepatocyte to transform into intracellular rounded forms, initiating the hepatic asexual development [45].

Preferably, the components in saliva that the sporozoites have adapted to and those in the mammalian environment that activate and maintain viability of the sporozoites can be combined in a buffer to preserve the quiescence of sporozoites, while still activating their motility needed to invade and traverse hepatocytes. Grace's mimicked the preservative environment of the salivary gland with its inorganic salt concentrations, while the addition of BSA and glucose prevented transformation, increased lifespan, and both activated and prolonged sporozoite motility. The combination resulted in enhanced viability of sporozoites over time as demonstrated by higher gliding of sporozoites in Grace's. Separately, the effect of glucose on prolonging *ex vivo* sporozoite viability was examined using two formulations of HBSS that differed only by glucose concentration. The *P. vivax* sporozoites behaved differently in the two HBSS formulations with viability generally improved in the HBSS with higher glucose concentration. However, *P. falciparum* sporozoites viability was comparably poor in both HBSS buffers. Based on this result with HBSS, glucose levels may need to be optimized in Grace's in future studies. In addition, future experiments should also investigate viability of sporozoites in more acidic variations of Grace's to further mimic the mosquito environment.

5. Conclusion

Overall both *P. vivax* and *P. falciparum* sporozoites in Grace's had better gliding at most time points except for sporozoites in RPMI at 0 hour. Interestingly, *P. vivax* sporozoites dissected into Grace's under optimal conditions in Thailand had a unique increase in gliding 4 and 8 hours post dissection, suggesting there was indeed a delay in activation of these sporozoites relative to the same sporozoites in the mammalian buffers and medium. These data are consistent with our hypothesis that a buffer more similar to the mosquito salivary gland microenvironment with the addition of factors

necessary for life and activation in the mammalian environment can enhance *ex vivo* sporozoite viability over time. The inorganic salt content of insect media such as Grace's provides the sporozoite with an environment more similar to the salivary gland than RPMI and HBSS. Simultaneously, the glucose and BSA provide the necessary factors for activating and prolonging the life span of *ex vivo* sporozoites. The use of Grace's and potentially other insect media for dissection may be able to significantly increase access to sporozoites, specifically *P. vivax* sporozoites. This prolonged viability allows for longer dissection times and the potential to ship dissected sporozoites with a decreased loss of viability. Additionally, access to sporozoites will be increased through enhanced viability of cryopreserved sporozoites dissected into insect media. This increased sporozoite access from the use of insect media will enable essential liver-stage studies to be conducted faster and more efficiently. This can help increase our understanding of the malaria parasite and give us greater opportunities to create more efficient drugs and effective vaccines as we combat the burden of malaria.

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Table 1

ANOVA relationship results.

Factor	Time	Time*Group	Buffer	Buffer*Group	Time*Buffer	Time*Buffer*Group
H₀ : No Effect (P-value)	<.0001 ^a	<.0001 ^a	<.0001 ^a	<.0001 ^a	<.0001 ^a	<.0001 ^a

a Denotes statistical significance of the relationship between factor and gliding percentage ($p < 0.05$)

Table 2

MGP's ($\bar{x}$ Gliding%) and statistical comparisons per buffer, time point, and experimental group.

Time (Hours)	Buffer	<i>P. vivax</i> - A					<i>P. vivax</i> - B					<i>P. falciparum</i>				
		$\bar{x}$ Gliding% (SE)	P-values			$\bar{x}$ Gliding% (SE)	P-values			$\bar{x}$ Gliding% (SE)	P-values					
			$\bar{x}$ = 0	$\bar{x}$ = RPMI	$\bar{x}$ = Grace's		$\bar{x}$ = 0	$\bar{x}$ = RPMI	$\bar{x}$ = Grace's		$\bar{x}$ = 0	$\bar{x}$ = RPMI	$\bar{x}$ = Grace's			
0	RPMI	57.42% (9.35) ^b	<.0001	N/A ^e	0.0001	31.64% (5.11)	<.0001	N/A ^e	0.1538	39.46% (14.55) ^b	<.0001	N/A ^e	<.0001			
	Grace's	43.93% (11.72) ^a	<.0001	0.0001	N/A ^e	32.65% (4.80)	<.0001	0.1538	N/A ^e	56.52% (8.56) ^a	<.0001	<.0001	N/A ^e			
	HBSS-1	58.28% (9.98) ^b	<.0001	0.7627	0.0001	11.19% (4.12) ^{a,b}	<.0001	<.0001	<.0001	31.89% (9.47) ^{a,b}	<.0001	0.0001	<.0001			
	HBSS-2	52.81% (12.12)	<.0001	0.1061	0.0252	17.71% (2.91) ^{a,b}	<.0001	<.0001	<.0001	45.02% (11.15) ^b	<.0001	0.0149	<.0001			
	PBS	7.23% (7.42) ^{a,b}	<.0001	<.0001	<.0001	0.00% (0.00) ^{a,b,c}	N/A ^d	<.0001	<.0001	15.55% (9.80) ^{a,b}	<.0001	0.0004	<.0001			
4	RPMI	34.96% (15.39) ^b	<.0001	N/A ^e	<.0001	0.29% (0.36) ^b	0.0002	N/A ^e	<.0001	0.31% (0.81) ^{b,c}	0.125	N/A ^e	<.0001			
	Grace's	56.23% (10.26) ^a	<.0001	<.0001	N/A ^e	11.72% (3.38) ^a	<.0001	<.0001	N/A ^e	37.37% (11.10) ^a	<.0001	<.0001	N/A ^e			
	HBSS-1	36.72% (11.48) ^b	<.0001	0.5298	<.0001	0.04% (0.14) ^{a,b,c}	0.5	0.0085	<.0001	7.81% (6.18) ^{a,b}	<.0001	<.0001	<.0001			
	HBSS 2	44.49% (11.22) ^b	<.0001	0.0173	0.0008	10.82% (3.23) ^a	<.0001	<.0001	0.4194	7.06% (3.33) ^{a,b}	<.0001	<.0001	<.0001			
	PBS	0.54% (1.42) ^{a,b,c}	0.25	<.0001	<.0001	0.00% (0.00) ^{a,b,c}	N/A ^d	0.0002	<.0001	1.67% (2.82) ^{a,b}	0.002	0.0098	<.0001			
8	RPMI	28.17% (15.88) ^b	<.0001	N/A ^e	<.0001	0.06% (0.15) ^{b,c}	0.125	N/A ^e	<.0001	0.00% (0.00) ^{b,c}	N/A ^d	N/A ^e	<.0001			
	Grace's	56.35% (9.94) ^a	<.0001	<.0001	N/A ^e	9.45% (2.74) ^a	<.0001	<.0001	N/A ^e	24.04% (15.18) ^a	<.0001	<.0001	N/A ^e			
	HBSS-1	34.37% (11.36) ^b	<.0001	0.0539	<.0001	0.03% (0.12) ^{b,c}	0.5	0.625	<.0001	2.55% (2.39) ^{a,b}	<.0001	<.0001	<.0001			
	HBSS-2	43.39% (13.11) ^{a,b}	<.0001	0.002	0.0013	4.35% (2.51) ^{a,b}	<.0001	<.0001	<.0001	1.67% (2.31) ^{a,b}	0.002	0.002	<.0001			
	PBS	0.00% (0.00) ^{a,b,c}	N/A ^d	<.0001	<.0001	0.00% (0.00) ^{b,c}	N/A ^d	0.125	<.0001	1.16% (3.05) ^{b,c}	0.125	0.125	<.0001			
24	RPMI	3.46% (5.21) ^b	0.001	N/A ^e	<.0001	0.00% (0.00) ^{b,c}	N/A ^d	N/A ^e	<.0001	0.00% (0.00) ^{b,c}	N/A ^d	N/A ^e	<.0001			
	Grace's	21.68% (14.74) ^a	<.0001	<.0001	N/A ^e	5.24% (3.18) ^a	<.0001	<.0001	N/A ^e	6.73% (4.83) ^a	<.0001	<.0001	N/A ^e			
	HBSS-1	11.87% (10.71) ^a	<.0001	0.0001	0.0338	0.02% (0.07) ^{b,c}	1	1	<.0001	0.86% (1.57) ^b	0.0313	0.0313	<.0001			
	HBSS-2	22.66% (13.52) ^a	<.0001	<.0001	0.8366	0.83% (0.80) ^{a,b}	<.0001	<.0001	<.0001	0.21% (0.93) ^{b,c}	1	1	<.0001			
	PBS	1.02% (1.88) ^b	0.0313	0.052	<.0001	0.00% (0.00) ^{b,c}	N/A ^d	N/A ^f	<.0001	0.19% (0.60) ^{b,c}	0.5	0.5	<.0001			

a Denotes statistical difference from RPMI $\bar{x}$ gliding % at respective time point ($p < 0.01$)

b Denotes statistical difference from Grace's $\bar{x}$ gliding % at respective time point ($p < 0.01$)

c Denotes statistical or numerical equivalence of $\bar{x}$ gliding % to 0 ($p < 0.05$)

d Test to determine statistical difference from 0 is not applicable when $\bar{x}$ gliding % equals 0

e Test to compare $\bar{x}$ gliding % to itself is not applicable

f Statistical test comparing equivalent means ($\bar{x}$) is not applicable

Fig. 1. Fields of gliding over time. Field of vision samples from RPMI and Grace's gliding assays at time 0, 4, 8, and 24 hours post dissection for each experimental group. MGPs were calculated by dividing the number of sporozoites with a gliding trail at least the length of the sporozoite by the total number of sporozoites in the field. Gliding percentages in bottom corners are average percentages from 20 fields of vision from the same assay. (A) *P. vivax*-A- Fields from gliding assays using sporozoites from mosquitoes infected with Thai isolates. (B) *P. vivax*-B- Fields from gliding assays using sporozoites from mosquitoes infected with Thai isolates and shipped from Thailand to the US for sporozoite isolation. (C) *P. falciparum*- Fields from gliding assays using sporozoites from mosquitoes fed in the US with *in vitro* gametocyte cultures.

Fig. 2. MGPs over time. Gliding assays were performed on sporozoites in RPMI, Grace's, two variations of HBSS and PBS at 0, 4, 8, and 24 hours post sporozoite isolation from anopheline mosquitoes. Each panel represents different experimental groups. In all panels, markers designate the MGP and error bars show standard error of the mean. (A) MGPs from group *P. vivax-A*, using sporozoites from mosquitoes infected with Thai field isolates. (B) MGPs from group *P. vivax-B*, using sporozoites from mosquitoes infected with Thai field isolates and shipped from Thailand to the US for sporozoite isolation. (C) MGPs from group *P. falciparum*, using sporozoites from mosquitoes fed in the US with *in vitro* gametocyte cultures.

Table A.1

	Ingredients	HBSS (mg/L)	RPMI 1640 (mg/L)	Grace's (mg/L)
Inorganic Salts	Potassium chloride (KCl) ^{abc}	400	400	4100
	Calcium chloride (CaCl ₂) (anhyd) ^b			750
	Magnesium chloride (anhyd) ^b			1070
	Potassium phosphate monobasic (KH ₂ PO ₄) ^{bc}	60		1013
	Sodium bicarbonate (NaHCO ₃) ^c	350		
	Sodium chloride (NaCl) ^{ac}	8000	6000	
	Sodium phosphate dibasic (Na ₂ HPO ₄) anhydrous ^{ac}	48	6000	
	Calcium nitrate (Ca(NO ₃) ₂ 4H ₂ O) ^a		100	
	Magnesium sulfate (MgSO ₄) (anhyd) ^{ab}		48.84	1358
Other Components	D-Glucose (Dextrose) ^{abc}	1000	2000	700
	D-Fructose ^b			400
	Fumaric acid ^b			55
	Succinic acid ^b			60
	Sucrose ^b			26680
	Malic acid ^b			670
	Alpha-Ketoglutaric acid ^b			370
	Glutathione (reduced) ^a		1	
	Phenol Red ^a		5	
Vitamins	Biotin ^{a b}		0.2	0.01
	Choline chloride ^{ab}		3	0.2
	D-Calcium pantothenate ^{ab}		0.25	0.02
	Folic acid ^{ab}		1	0.02
	Nicotinic acid (niacin) ^b			0.02
	Niacinamide ^a		1	
	Para-Aminobenzoic acid ^{ab}		1	0.02
	Pyridoxine hydrochloride ^{ab}		1	0.02
	Riboflavin ^{ab}		0.2	0.02
	Thiamine hydrochloride ^{ab}		1	0.02
	Vitamin B12 ^a		0.005	
	i-Inositol ^{ab}		35	0.02
Amino Acids	Glycine ^{ab}		10	650
	L-Alanine ^b			225
	L-Arginine hydrochloride ^b			700
	L-Arginine ^a		200	
	L-Asparagine ^{ab}		50	350
	L-Aspartic acid ^{ab}		20	350
	L-Cystine 2HCl ^{ab}		65	28.68
	L-Glutamic acid ^{ab}		20	600
	L-Glutamine ^{ab}		300	600
	L-Histidine ^{ab}		15	2500
	L-Hydroxyproline ^a		20	
	L-Isoleucine ^{ab}		50	50

L-Leucine ^{ab}	50	75
L-Lysine hydrochloride ^{ab}	40	625
L-Methionine ^{ab}	15	50
L-Phenylalanine ^{ab}	15	150
L-Proline ^{ab}	20	350
L-Serine ^{ab}	30	550
L-Threonine ^{ab}	20	175
L-Tryptophan ^{ab}	5	100
L-Tyrosine disodium salt ^b		62.14
L-Tyrosine disodium salt dihydrate ^a	29	
L-Valine ^{ab}	20	100
beta-Alanine ^b		200

a Ingredient is in RPMI

b Ingredient is in Grace's

c Ingredient is HBSS

Graphical abstract