Modelling input-output relationships

to optimize production of a live-attenuated malaria vaccine

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Modelling input-output relationships to optimize production of a live-attenuated malaria vaccine (paper)

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Abstract The production process for any vaccine requires thorough characterization of inputs and outputs. This profile allows a production facility to closely monitor the resource efficiency and batch quality of the given vaccine. Good product characterization is especially important for adherence to cGMP. These standards similarly apply to the ongoing development of a live-attenuated malaria vaccine. The research presented in this paper explores the relationship between gametocyte exflagellation as an input and malaria infection in mosquitoes as an output, quantified by the number of oocysts produced in the mosquito. The goal of this research was to develop a deterministic model to capture the relationship between these data and to measure the influence of other parameters, such as gametocytemia and sex ratio, on infection. However, due to the weakness of the relationship between the process inputs and output (with an $R^2 < 0.75$ across all models), the deterministic model cannot be produced. The paper discusses several factors which might account for this weak relationship, some of which include the influence of unmeasured inputs, large error around the input measurements, and a limited data set size. Future work which overcomes these limitations can produce a model which predicts oocyst output using the process inputs.

Introduction

Malaria is one of the oldest, most lethal diseases to affect humanity. The most endemic strain is caused by the apicomplexan parasite, *Plasmodium* falciparum, and transmitted by mosquitoes of the genus *Anopheles*. While health interventions over the last several decades have drastically improved outcomes of the disease, much of the developing world still suffers from it. In 2019, 229 million cases were reported globally with over 93% occurring in Sub-Saharan Africa [1, 2]. Unfortunately, the disease is particularly lethal for children. That same year saw 409,000 related deaths, and two out of three victims were under the age of 5 [1, 2]. Furthermore, climate change threatens to expand the global area in which malaria is transmissible [4]. In addition to the compelling humanitarian case, the disease costs over $12 billion annually and incalculable losses in human capital. The world has coalesced around several potential interventions to slow the spread of malaria and eventually eradicate it. Vaccines are a key goal in this effort.

Humanitarian organizations around the world are committing over $3 billion annually to the development of a malaria vaccine. Historically, the path to the vaccine has been hampered by the biological complexity of *P*. falciparum [5-7]. However, recent work has culminated in a new live-attenuated vaccine which uses a specific life stage of the parasite--the sporozoite stage--as the active ingredient [8]. While this is enormous progress, the malaria vaccine still faces some challenges. Today, scale-up and resource efficient production are among the greatest obstacles to widespread distribution of the vaccine. This research project aims to address a corner of the production roadblocks. By characterizing the relationship between the inputs and outputs of the sporozoite production process, we can better control conditions for sporozoite cultivation. This will hopefully lead to a high yield, scalable upstream process for the vaccine.

Background

Malaria transmission and the *P*. falciparum life cycle. Malaria begins in humans as the sporozoite

stage of the apicomplexan parasite, genus *Plasmodium*. While there are many species of *Plasmodium* which cause different types of malaria, This research focuses on *P*. falciparum (*P*f) which accounts for 92% of malaria cases globally [1]. *P*f sporozoites enter the human body with mosquito saliva when a person is bitten by an infected female *Anopheles* mosquito. The most viable sporozoites cross into the bloodstream from the injection site and travel to the liver to invade hepatocytes [18-20]. Through a process called cell traversal, sporozoites cut through several hepatocytes before settling in one [15, 20] although the mechanism which governs this switch between traversal and settling is currently unknown. Once they have invaded a hepatocyte, they quietly replicate in structures called schizonts, hidden away from the immune system while consuming the host cell. After 10-14 days, these schizonts rupture to free the next developmental stage: *P*f merozoites [22].

Merozoites are responsible for the symptomatic stage of malaria as they flood the circulatory system to invade and destroy red blood cells, also called erythrocytes. This process, called the erythrocytic phase of infection, is a form of asexual replication. The merozoites replicate in the host cell before rupturing it, then repeat the process with neighboring cells. Eventually, through a currently unknown signal, some merozoites abandon asexual reproduction and instead, begin producing male and female gametocytes for sexual reproduction [27]. These gametocytes mature from their first stage (I) to final stage (V) over 14 days. This development stage is necessary to seed infection in the next mosquito to bite.

When a mosquito bites an infected human, it ingests *P*f gametocytes with the blood it intended to consume. As temperature and pH decrease from the human circulatory system to the mosquito midgut, gametocytes respond by transforming into gametes and beginning reproduction. These conditions and the presence of a mosquito metabolite, called xanthurenic acid, induce a process called exflagellation [5, 9]. During exflagellation male gametes extend flagella in all directions in an attempt to fertilize female gametes. Together, they produce zygotes which become ookinetes, the next stage for amplifying the mosquito infection. The ookinetes cut through the cells of the midgut epithelium in another instance of cell traversal [5, 10]. They deposit themselves on the basal lamina of the mosquito midgut and form oocysts, sac-like structures for parasitic replication. Over 7-10 days, these oocysts incubate sporozoites while taking nutrients from the host. Once developed, the sporozoites rupture the oocyst sac and migrate to the mosquito salivary gland to begin a new infection cycle in another human [5, 17, 18].

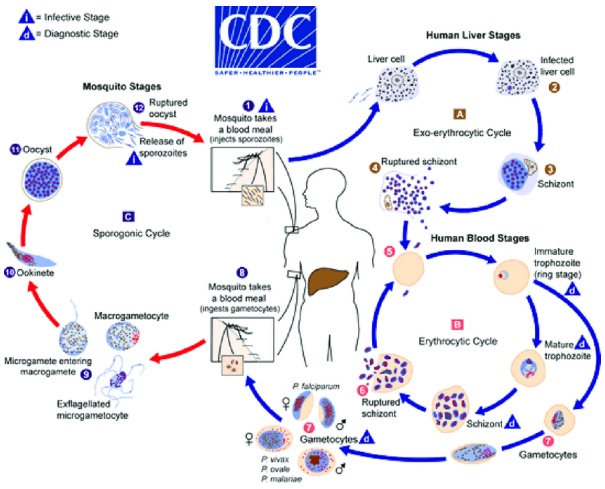

Figure 1. The life cycle of Plasmodium falciparum takes place across two hosts: the human and the *Anopheles* mosquito. The figure above captures this life cycle in detail, summarizing how the disease is transmitted from host to host [28].

Bottlenecks in transmission. This complex transmission process between mosquitoes and humans comes with several bottlenecks to *P*. falciparum development. In mosquitoes, the parasite is most vulnerable any time from when it is consumed until it creates oocysts on the midgut. The ookinete population in the mosquito is linearly related to the number of gametocytes consumed; similarly, the number of oocysts produced is less than or equal to the number of ookinetes formed. These bottlenecks make the sex ratio of the injested gametocytes and the immune response of the mosquito incredibly relevant to

successfully seeding infection [5, 13]. These bottlenecks are points of control on sporozoite development. They are incredibly important for the future industrial-scale production of the malaria vaccine as well as other health interventions for malaria.

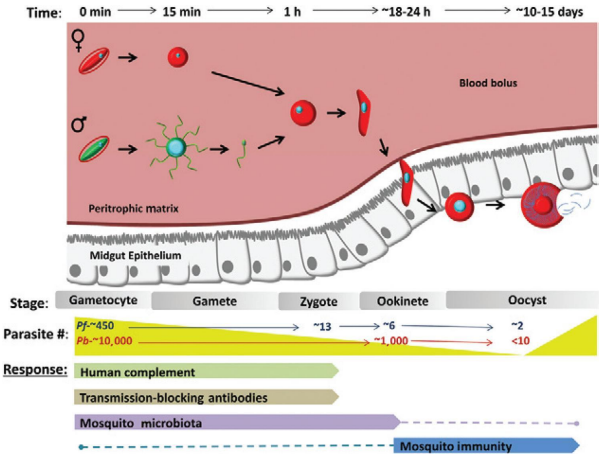

Figure 2. The figure above illustrates *P.* falciparum development from gametocyte to oocyst and the time each step takes. As illustrated, *P.* falciparum sees a near 100x population reduction between fertilization and ookinete formation. The population only rebounds after the oocysts have ruptured to produce sporozoites [13].

Developing the PfSPZ vaccine. In humans, infection is bottlenecked at sporozoite injection. This step became the ideal intervention point for the PfSPZ vaccine, a live-attenuated malaria vaccine produced by Sanaria. Sporozoites are potentially the perfect target for training an immune response because they do not produce symptomatic infection until they have developed into merozoites in the liver. For this reason, the active pharmaceutical ingredient of the PfSPZ vaccine is the attenuated sporozoite. Even when attenuated, the parasite can cross into the circulatory system; however, it will be too damaged to develop into merozoites if it manages to navigate to the liver. The motility of the attenuated sporozoite becomes the key factor in assessing the potency of the vaccine.

The PfSPZ vaccine was based on the results of a trial which used attenuated sporozoites to inoculate participants against malaria [24]. Fourteen volunteers entered a trial which measured the effects of this form of inoculation. Over 5-19 sessions, *Anopheles* mosquito bit the participants, exposing them to 100,000-300,000 attenuated sporozoites in total. The sporozoites were attenuated with 15,000 rad of gamma radiation. This irradiation damages them enough to prevent replication into merozoites while preserving their motility [8]. This experiment format replicates the ideal conditions for sporozoite development and invasion as *P.* falciparum co-evolved with *Anopheles* as its vector for infection. The trials proved promising, conferring 93% protection against infection during a challenge trial conducted 2-10 weeks later [8]. However, given the experimental setup, this degree of protection has proven difficult to scale for industrial production.

Several factors make the high immunity demonstrated in the trial difficult to achieve. The trial used mosquitoes to inoculate participants with the attenuated sporozoite; however, mosquito injection is not practical or comfortable enough for commercial use. A viable live-attenuated malaria vaccine would require the sporozoites to be extracted from the mosquito (or grown *in vitro*) and delivered intradermally or intravenously. These artificial methods for injection potentially reduce the potency of the sporozoites because they are no longer adapted to the delivery mechanism. The vaccine manufacturer must develop methods for producing and preserving the sporozoites before injection. The authors of the trial found that a potential vaccine, loaded with cryopreserved sporozoites which are delivered intradermally, could require 4-16 times more sporozoites to confer immunity [8]. Even this estimate assumes that sporozoites are efficiently cultivated with low production loss. Thus, a single treatment could require over 1,600,000 attenuated sporozoites. The authors of the trial estimated that a team of 6 skilled workers would take one hour to harvest enough sporozoites for 500 vaccine doses (84 doses/worker/hour) [8]. If this team worked continuously through the 8760 hours in a year, they could only produce 4.38 million vaccines compared to the over 220 million cases of malaria

annually. Fifty teams of 6 could meet this demand under the same conditions; however, this scenario assumes a perfect matchup between each dose of vaccine and each person who would have contracted malaria without it. We are also assuming that only one dose will be required to build immunity which is unlikely [8].

This challenge raises the need for better process characterization. This research thesis aims to build on previous work by advancing characterization of the upstream production process for the live-attenuated vaccine. Several *in vitro* and *in vivo* techniques have been linked together to develop a process for manufacturing sporozoites. The process starts when merozoites enter their sexual stage, producing gametocytes *in vitro* [27]. These gametocytes mature to stage V, then they are fed to mosquitoes [27]. *In vivo* the gametocytes develop into oocysts, then eventually migrate to the salivary gland as sporozoites [8]. Individually, these steps contain significant biological variation. When appended together, this noise propagates through subsequent steps, making parameter analysis particularly difficult. Fully characterizing each step will allow a future, industrial-scale process to control error and ensure quality output. This research thesis is concerned with characterizing the relationship between the inputs and outputs of the sporozoite production process.

Methods

Outline of experiments. In the lab, *P*. falciparum develops across three key steps. First, trophozoites (ring stage before asexual reproduction) from a source culture seed gametocytes in a new culture. Next, the gametocytes mature from stage I to stage V. Finally, gametocytes are fed to mosquitoes to produce sporozoites *in vivo*. The results discussed in this paper are primarily concerned with the final step in this process. By measuring how the inputs to mosquito feeding affect the outputs of the process, we can set appropriate targets for the rest of the system. This is a type of top-down approach which sets the bounds within which the input can deliver a high product yield.

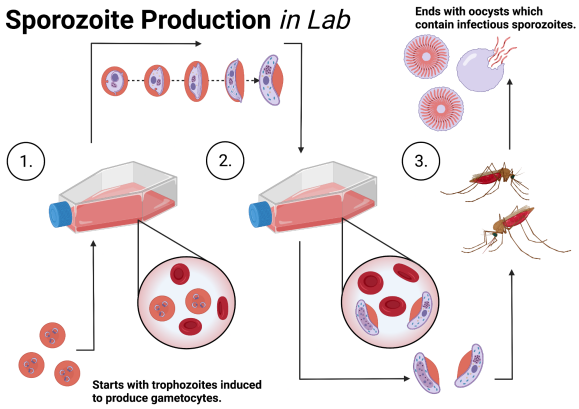

Figure 3. The figure above diagrams the three steps to producing sporozoites in the lab. 1) Trophozoites (ring stage), which are illustrated in the bottom-left, enter a new culture flask and are induced into gametocytes. 2) The gametocytes mature from stage I to V in the culture flask. 3) The gametocytes are fed to mosquitoes during a process called standard membrane feeding assay (SMFA). The final products are oocysts, illustrated in the top-right corner. *Created by the author with BioRender.com.*

The final step in the cultivation process has been divided into three experiments: measuring gametocytemia, measuring percent exflagellation, and the standard membrane feeding assay (SMFA). Gametocytemia is the percentage of cells in a sample that are stage V *Pf* gametocytes. Percent exflagellation is the percentage of cells in a sample that can exflagellate which is equal to the number of mature male gametocytes. SMFA is a standardized technique for feeding gametocytes to mosquitoes. Each of these experiments will be discussed in detail in the subsequent sections. These experiments produce three key data points which inform the results in this paper: gametocytemia (%), exflagellation (%), and infectivity respectively. The methods used to conduct each experiment have been described here.

Overview of conditions leading up to the experiments. The gametocytes were seeded from the asexual strains NF54, NF54-Transgenic (G8), NF135, and APL5G. It is important that the asexual seed culture has a high sexual conversion rate. This factor governs how frequently they

produce gametocytes *in vitro*, and it decreases as the culture ages [7]. The gametocytes developed in a cell culture flask at 37.0 °C mixed into a growth medium composed of human serum and blood. The human serum is filtered and sterilized before it is used in growth medium. This step is important and has been covered extensively in previous work [27]. The potential of the new batch of human serum to produce exflagellating gametocytes should be empirically tested against a control batch with known production potential [27]. Gametocytes are extremely sensitive to this component of the growth medium, so this testing minimizes the effect of changing serum on gametocyte production.

The blood and serum must also have compatible blood types for healthy gametocyte development. Gametocytes are cultured in O+ or A+ blood based on availability. However, O+ blood in particular seems to be the most productive type for development [29]. Gametocytes are also sensitive to the age of the erythrocytes. Ideally, culture flasks are only fed with erythrocytes that are less than 2 weeks old. The erythrocytes can be stored for this long in a refrigerator at 4 °C [27]. These conditions produced the cell cultures which are discussed in this paper.

Mosquito feeding and SMFA. Mosquitoes are the natural vector for malaria transmission. Thus, they are an ideal incubator for lab-grown sporozoites because they abstract away the complexity of sporozoite development. However, the mosquitoes must be incredibly similar within and between batches in order to be treated as a blackbox. The mosquitoes used to produce these results are all raised together under the same conditions under an identical diet of starch, water and antibiotics. These mosquitoes grow from pupa to adult mosquitoes in 7-14 days. Then, the female mosquitoes are separated from the rest of the population to be used in the feed. The female mosquitoes that mated before being separated will produce eggs after feeding while the virgin mosquitoes will not produce eggs; however, these females cannot be superficially distinguished at this point. Because this distinction will only be discovered during dissection, mated and virgin mosquitoes are combined into groups, or pots, for feeding. Each pot, containing approximately 25 mosquitoes, will receive only one gametocyte culture.

These pots are fed through a process called SMFA. SMFA is a standard set of techniques through which stage V gametocytes are fed to mosquitoes in an attempt to induce infection. SMFA is meant to resemble how infection propagates in real life [27]. Each feed tests the infectivity of several gametocyte cultures by feeding a single pot of mosquitoes with a single culture. The cultures are diluted down to 0.1% gametocytemia and fed to mosquitoes using an artificial membrane feeding device. This setup emulates the warm body temperature and skin-like texture which attract mosquitoes for natural feedings. Then, the pots are starved for 2 days to kill any mosquitoes that did not consume blood.

After 7-10 days, several mosquitoes from each pot are dissected to count oocysts on their midguts. The exact day within this range does not matter because we will see evidence of oocyst formation regardless of whether the oocyst is premature (in the case that we looked too early) or has been ruptured (in the case that we looked too late). At dissection, mosquitoes are also separated into two groups: an egg group and virgin group. The oocyst count of each midgut is translated into several metrics, including an average oocyst count per midgut for each group, a median oocyst count, a standard deviation and a percent “infectivity”, the percentage of mosquitoes with at least one oocyst. We also collected data on whether or not the mosquito was carrying eggs at the time of dissection. Together, these metrics characterize how effective a gametocyte culture is in producing infection in its pot. While SMFA is regarded as a low-throughput screening technique, it is also a “gold-standard” convention [27] for inducing infection in mosquitoes.

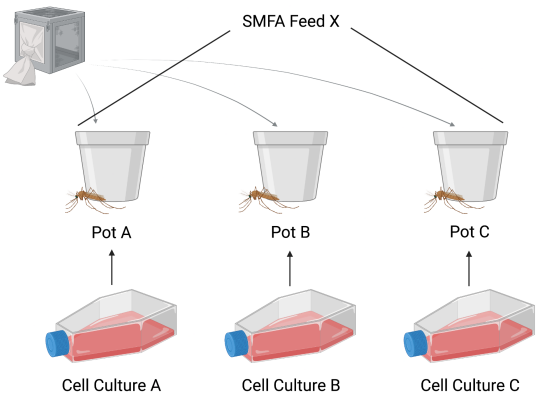

Figure 4. This illustration depicts how gametocyte cultures are allocated to mosquito pots within an SMFA. The mosquito insectary is depicted in the top-left corner. All mosquitoes used in the feed are sourced from the same insectary and raised under the same conditions. Each pot will contain roughly 25 mosquitoes of 70-80 total mosquitoes in the feed. *Created by the author with BioRender.com.*

Measuring exflagellation. Percent exflagellation, or simply exflagellation, assesses the population of viable male gametocytes in a culture before SMFA. To begin the measurement, a sample must be prepared from the culture flask. First, 30 µL of ookinete solution is removed from the refrigerator which has been storing it at 4 °C. The ookinete solution used for these experiments was not prepared by the author; however, its preparation instructions have been outlined in previous work [27]. This 30 µL of ookinete solution is mixed with a 30 µL sample of the gametocyte culture taken from 37.0 °C. The gametocytes should be gently stirred before sampling. This ensures that enough cells are collected to observe exflagellation. Once combined, the 60 µL sample is pipetted on a hemocytometer. Immediately, a timer is started for 15 minutes, the recommended point from which to begin measuring exflagellation.

The hemocytometer is divided into a counting region in each corner and one in the center as depicted in **figure 5**. At 15 minutes, exflagellation events are counted under a microscope at 40x objective in each corner region and recorded. Exflagellation events can be identified by the rapid shaking of several cells around some focal point. Counting these events takes approximately 15-20 minutes. This step is extremely time-sensitive because the male gametocytes die several minutes after they start exflagellation *in vitro*. Once this count has been completed, red blood cells (RBC) are counted from the central region of the hemocytometer. In this region, RBCs are only counted from four grid cells at each corner of the center region. These RBC counting regions are depicted in blue in **figure 5**. RBC counting is not time sensitive and thus, can proceed at any pace.

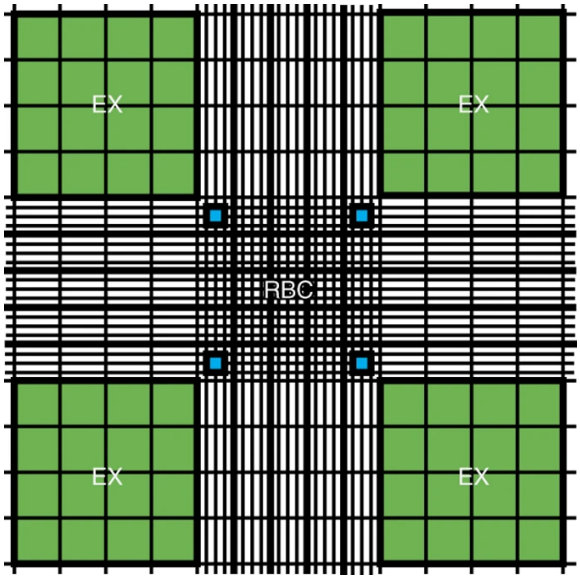

Figure 5. This is an illustration of a hemocytometer with the counting regions labelled [27]. The exflagellation counting regions are depicted in green while the RBC counting regions are depicted in blue.

Calculating percent exflagellation. Through the equations below, our measurements are converted into percent exflagellation [27]. The average of our exflagellation counts in each corner is $\mu_{exflag.\,events}$; the average of our RBC counts in each corner of the central grid is $\mu_{RBC\,count}$; and $f_{dilution} = 2$ represents the dilution factor.

$$\mu_{exflag.\,events} * f_{dilution} * 10^4 = exflag.\ per\ mL \quad (1)$$

$$\mu_{RBC\,count} * f_{dilution} * 100 * 10^4 = RBC\ per\ mL \quad (2)$$

$$\frac{exflagellation\ per\ mL}{RBC\ per\ mL} * 100 = exflagellation\ (\%) \quad (3)$$

Measuring gametocytemia. Gametocytemia reflects the percentage of cells that are stage V gametocytes in a sample. Before taking the measurement, a giemsa smear slide must be prepared with cells from the culture flask. First, a clean slide is brushed with a cell sample from the gametocyte culture flask. Then, the slide is dipped in methanol to suspend the cells. Finally, the slide is submerged in giemsa to dye the cells. This step makes the gametocytes especially visible in the red blood cells. Allow the slide to sit for 3 minutes before removing and rinse with water. The giemsa dye was not prepared by the author. Instructions for its preparation have been discussed extensively in previous work [27].

Once the slide is ready, it is placed under a microscope at 40x objective. A lens with a 10x10 grid overlaid on its view helped standardize counting methods across experiments (**figure 6**). Red blood cells are counted only in the one column of the grid. Any column can be selected; however, this choice cannot change between optical views. Then, without moving the grid, all stage V gametocytes within the grid are counted. The sex of the stage V gametocytes can also be observed during this process. Male gametocytes can be differentiated from females by the compactness of their nuclei. Male gametocytes usually have more dispersed nuclei than female gametocytes do. These steps are repeated by randomly moving the field of view until at least 350 red blood cells have been counted.

Calculating gametocytemia. The equation below converts our red blood cell count and gametocyte count into the gametocytemia of the culture.

$$\frac{gametocyte\ count}{RBCcount * 10} * 100 = gametocytemia\ (\%)\ (4)$$

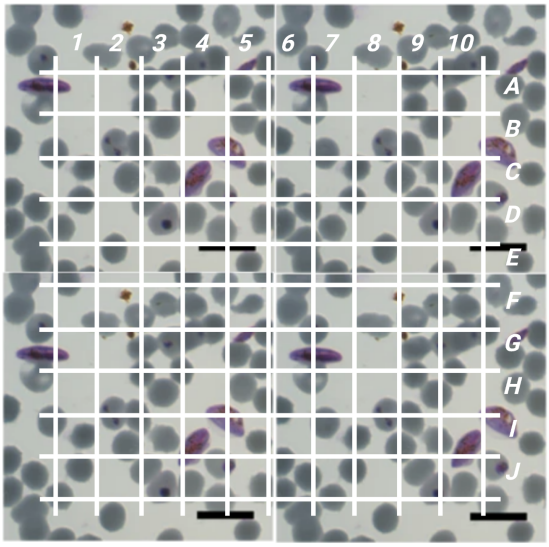

Figure 6. This figure depicts a typical field of view of the sample under the microscope. Notice the 10x10 grid overlaid on the sample. Red blood cells will only be counted from one column while all gametocytes in the grid will be counted before moving the field of view. The gametocyte image is a permutation of the original [27]. *Created by the author with BioRender.com.*

Labelling gametocyte cultures. Each gametocyte culture was labelled to track the relationship between gametocytemia, exflagellation and infection output. The age of the gametocyte culture is the number of days between the date that culture was seeded and the date that it was fed to mosquitoes during SMFA. Each experiment is also labelled by its parasite strain and a feed number. The feed number indicates which SMFA batch contains a pot that was infected by the culture. Finally, the gametocyte cultures are sometimes labelled with metadata about which asexual culture seeded it or who cultured it. This information was often added to differentiate multiple cultures of the same parasite strain within the same SMFA batch. See **supplementary information** for more details.

Modelling techniques. The modelling techniques in this research paper were selected with some assumptions about the data and its relationships. From the previously discussed experimental data, three key relationships were modelled in this paper: gametocytemia against exflagellation, the effect of exflagellation on infection, and the effect of sex ratio on infection. In each case, the axes

have been set to a minimum of 0 as none of these values can be negative. For infectivity, the axis was capped at 100 to reflect that percentage of mosquitoes infected cannot exceed 100%. Oocyst output is a discrete variable while the others have been treated as continuous. Finally, the egg group mosquitoes are modelled separately from the virgin group. This choice allows for comparisons between both groups.

A linear regression was used to model gametocytemia against exflagellation, sex ratio against average oocyst output, and exflagellation against average oocyst output. Linear regression was chosen because of its simplicity and usefulness for exploring an unknown relationship between a single input and a single output. Therefore, the model for gametocytemia against exflagellation inherently assumes that gametocytemia is the only significant input affecting exflagellation. Similarly, the model for exflagellation assumes exflagellation is the only or most significant input affecting average oocyst output. We must also acknowledge that the model was built with a fairly small data set of fewer than 30 experiments; whereas, a more robust model would consider hundreds or thousands of experiments. This is primarily a limitation of time because a single feed takes approximately 4 weeks to complete from culture seeding to oocyst production. Furthermore, the data set does not contain any experiments that share similar gametocytemia values while having different exflagellations; nor does it contain any experiments that share similar exflagellations while having different average oocyst outputs. This limitation is due to the size of the data set and the fact that these input values are continuous rather than discrete. This issue limits how well we can detect biological noise around each experiment.

A logarithmic regression was used to model sex ratio against infectivty and exflagellation against infectivity. A logarithmic model offers the advantage of setting an upper limit on infectivity. Given that infectivity is the percentage of midguts infected from the pot, it cannot exceed 100%.

Results

The figures below were generated with data gathered throughout this research project. The raw data can be accessed through **supplementary information** along with some background as to how the graphs were generated. Each point on the scatter plots represents a single experiment. Results from the egg group mosquitoes are separated from that of the virgin mosquitoes. The figures have had intercept values set to 0. Data was gathered for feeds 13-20; however, feeds 13 and 14 were omitted from the results. The experiments from these feeds were conducted with a focus on learning rather than rigorous data collection. There are also some gaps in the data from 13 and 14 which prevent them from being included in the figures below. Additionally, data on gametocyte sex ratios was only collected from feed 18 onwards. Sex ratio was a late consideration as a potential variable to explore.

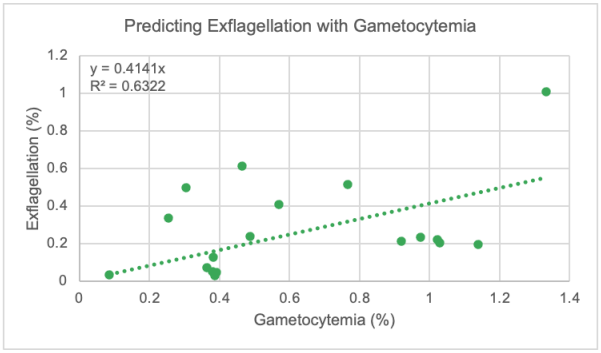

Figure 7. *Exflagellation against gametocytemia* (Feed 15-20). Each point represents an experiment where the gametocytemia and the exflagellation of the culture were measured. A linear trendline was drawn, producing an R^2 of 0.6322.

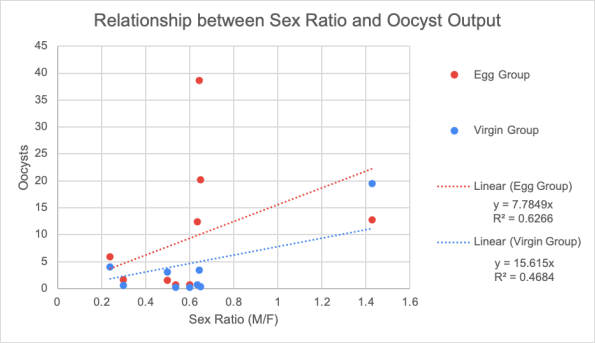

Figure 8. *Sex ratio against average oocyst output* (Feed 18-20). Sex ratio is defined as the number of male gametocytes to female gametocytes. The sex ratio of each culture is plotted against its average oocysts output. The egg group mosquitoes are graphed in red (R^2 = 0.6266) while the virgin group mosquitoes are graphed in blue (R^2 = 0.4684).

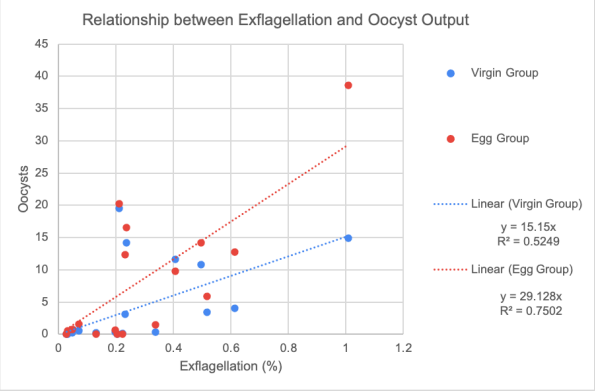

Figure 9. *Exflagellation against average oocyst output* (Feed 15-20). The figure plots the exflagellation of each experiment against its average oocyst output. The average oocyst output is the average number of oocysts produced by a mosquito in the given pot. The egg group mosquitoes are graphed in red (R^2 = 0.7502) while the virgin group mosquitoes are graphed in blue (R^2 = 0.5249).

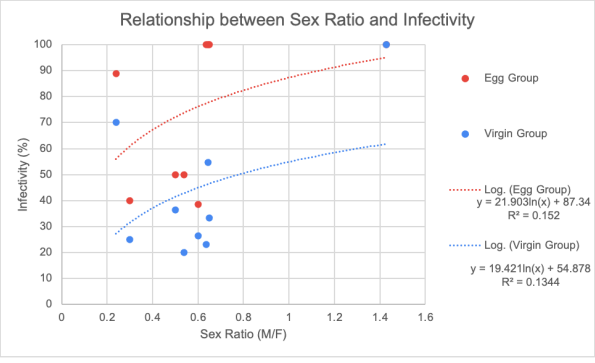

Figure 10. *Sex ratio against infectivity* (Feed 18-20). The figure plots the sex ratio of each experiment against the infectivity that it produced in its given pot. The egg group mosquitoes are graphed in red (R^2 = 0.152) while the virgin group mosquitoes are graphed in blue (R^2 = 0.1344).

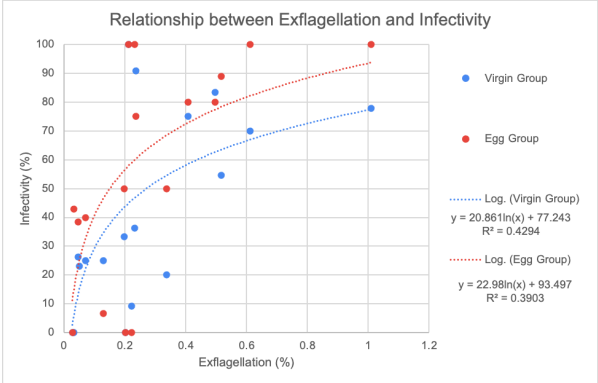

Figure 11. *Exflagellation against infectivity* (Feed 15-20). The figure plots the exflagellation of each experiment against the infectivity that it produced in its given pot. The egg group mosquitoes are graphed in red (R^2 = 0.3903) while the virgin group mosquitoes are graphed in blue (R^2 = 0.4294).

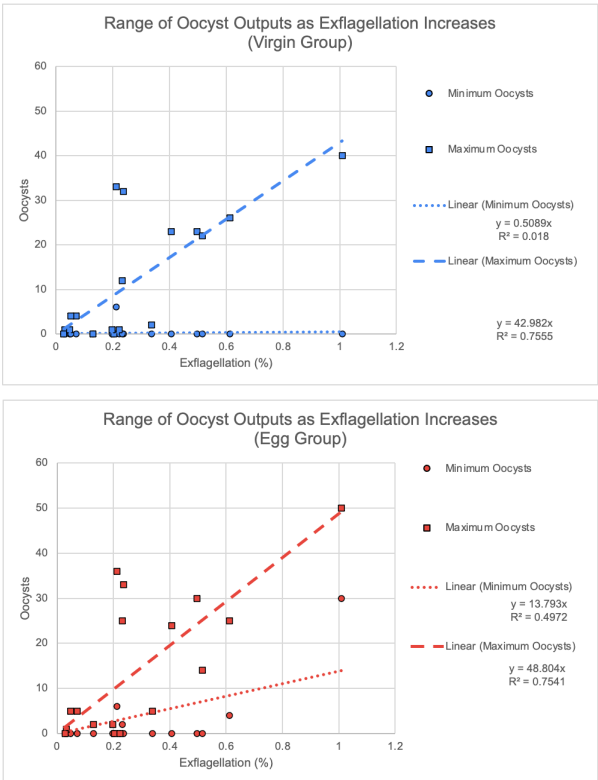

Figure 12. *Exflagellation against range of oocyst output in virgin group* (top) and *exflagellation against range of oocyst output in egg group* (bottom) (Feed 15-20). The figures depict how the range of oocysts produced in a given pot varies as exflagellation changes. The maximum points have been identified as square points and the minimums are round points. For the virgin group, the minimum oocyst outputs produced a trendline of y=0.5089x (R^2 = 0.018), and the maximum oocyst outputs produced a trendline of y=42.982x (R^2 = 0.7555). For the egg group, the minimum oocyst outputs produced a trendline of y=13.793x (R^2 = 0.472), and the maximum oocyst outputs produced a trendline of y=48.804x (R^2 = 0.7541).

Discussion

While the results confirm a relationship between the inputs and outputs, the significance of the relationships in all cases is too weak to produce a deterministic model. While the original aim of the project cannot be achieved with the given data, several insights can be drawn to move closer to constructing such a model.

Controlling error in oocyst output. The high statistical error of oocyst output (see **supplementary information** for **tables 1** and **2**) shows that many elements of the sporozoite production process are still uncontrolled. This error is most visible when comparing the standard deviation around the oocyst output of a pot to its average oocyst output per mosquito. We can observe this phenomenon by looking at the standard deviation across experiments.

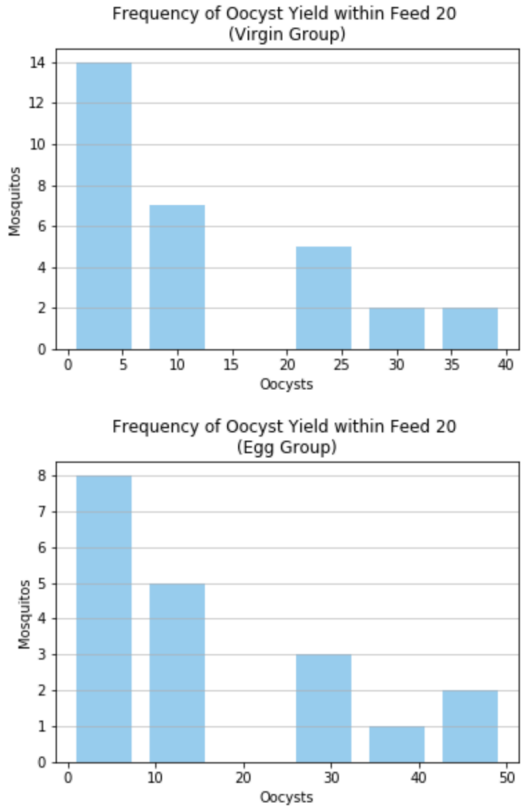

Figure 13. The top figure shows the frequency of oocysts outputs in the virgin group from Feed 20 while the bottom figure reflects the same in the egg group. This distribution includes all 48 mosquitoes in the feed, regardless of gametocyte culture consumed.

For the egg group, this value is 96% of the average. For the virgin group, it is 140% (Feed 13-20). The standard deviation is so high because of the distribution of oocysts recovered from mosquitoes in a pot. **Figure 13** demonstrates this by investigating this phenomenon in Feed 20. For most feeds, the vast majority of mosquitoes produce fewer than 10 oocysts. This is again reflected in the fact that fewer than 32% of experiments recorded an average oocyst output greater than 10 (see **tables 1**, **2**). However, many feeds also seem to contain a few mosquitoes with incredibly high infection (> 25 oocysts). These mosquitoes cannot be considered outliers as this pattern recurs across many of the feeds where infectivity > 49%. A few factors might be responsible for this distribution of oocyst output.

While the mosquitoes are consuming the same volume of blood [31], they might not be consuming the same concentration of gametocytes. This might be the reason why there are a few mosquitoes with incredibly high infection and many with little or no infection. The first few mosquitoes to feed on the gametocyte culture during SMFA might be consuming blood with a high gametocytes concentration. When the rest of the mosquitoes get an opportunity to feed, the concentration of infectious gametocytes in the sample might have decreased substantially. Because they have still consumed blood, they do not die when the pot is starved for 2 days, and they avoid infection. This might account for the large range of infection results where the majority of mosquitoes see little to no infection while a few mosquitoes see very high infection.

Controlling error in exflagellation. Another significant point of error is percent exflagellation. This measurement technique faces two primary issues. First, its accuracy relies significantly on the skills and choices of the person taking the measurement. This person must make decisions about whether or not an exflagellation event falls within the counting region on the hemocytometer depicted in **Figure 5**. This decision is made, all while the cells are moving between borders. The

rules for counting exflagellation are decided by the individual because they allow the person to compare experiments that were recorded on different days. Unfortunately, these decisions also make it incredibly difficult for anyone else to replicate those measurements. A deterministic model would need to be built on experiments that can be replicated on identical samples.

Furthermore, exflagellation in male gametocytes is an extremely time-sensitive process. Male gametocytes die *in vitro* after only a few minutes exflagellating. This happens while other gametocytes in the same sample do not begin exflagellating until well past the 15-minute start time. These late exflagellation events might occur in a section of the hemocytometer which has already been examined. For each exflagellation measurement, the number of events in each corner of the hemocytometer were recorded. **Figure 14** shows how gametocyte death affects the number of exflagellation events counted in each quadrant.

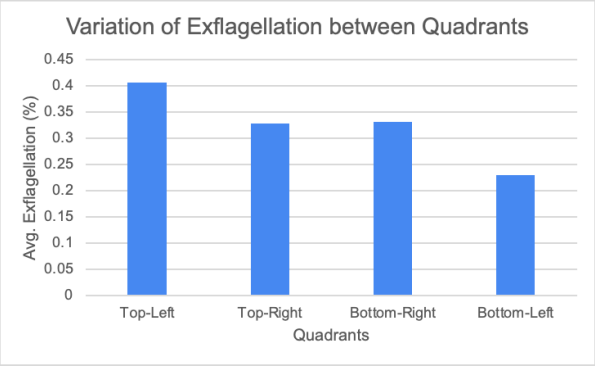

Figure 14. The figure shows the average of the exflagellation measurements if only that given quadrant was considered. The values here account for differences in the sample cell density between experiments. The bars are depicted in their counting order, from left to right.

Gametocytes were counted in the following order for all experiments: (1) top-left, (2) top-right, (3) bottom-right, (4) bottom-left. In **figure 14**, we see that exflagellation trends downward as we move from quadrant to quadrant. Gametocytes seem to die before they are counted, especially those in the final quadrant: bottom-left.

Controlling error in gametocytemia. A similar type of error occurs with gametocytemia. Since gametocytemia is measured manually, the same sample is prone to vary between experiment facilitators; however, gametocytemia is not time sensitive at all. This frees the measurement from some of the major error issues associated with exflagellation measurements.

Gametocytemia against exflagellation. Figure 7 shows that there is a significant relationship between gametocytemia and exflagellation. The linear model ($R^2 = 0.6322$) demonstrates that as gametocytemia increases, exflagellation generally increases. These findings align with the understanding that both measurements represent a form of parasitemia in the sampled system. While gametocytemia includes mature male and female gametocytes, exflagellation specifically accounts for male gametocytes that can exflagellate.

Despite the obvious correlation, the R^2 is too weak ($R^2 < 0.98$) to build a deterministic model of this relationship. One possible reason for the weakness of this relationship is that there might be one or several unmeasured inputs to the system. We know that time affects exflagellation because it is an event-based measurement. As time increases, exflagellations within the system might be at one of several stages of occurring (starting, happening, or ending). These individual exflagellation events sum to the percent exflagellation of the system itself. While all experiments are completed roughly in the same timeframe (30-35 minutes total), the difference of several seconds to a minute between experiments might affect the strength of the model. Time would likely need to be considered as a variable to improve the R^2 of this model. We should expect time to have very little influence until at least 29 minutes have passed since the culture was removed from its incubator. With multiple inputs and much more data, we might also find that the relationship between gametocytemia and exflagellation is non-linear. That would suggest that as the gametocyte population in the sample increases, the gametocytes have some effect on

the exflagellation of neighboring gametocytes. There are still many unknowns concerning *P. falciparum* signalling, and it is believed that neighboring parasites perform some form of cell signalling when committing to their sexual stage from their asexual stage [27]. It is possible that some form of signalling is occurring here as well.

Egg group vs. virgin group. The results show that as exflagellation increases, the number of oocysts produced in both the egg group (R^2 = 0.7502) and the virgin group (R^2 = 0.5249) increases. Furthermore, the egg-carrying mosquitoes generally produced more oocysts than the virgin mosquitoes, approximately 4 more oocysts on average. It is also noteworthy that the R^2 of the egg group was significantly higher than that of the virgin group. This is because baseline infectivity in most cases was higher among egg-carriers than it was among virgin mosquitoes. Several factors might explain this gap in infection.

Previous work has demonstrated that there are significant biological and hormonal differences between egg-carrying mosquitoes and virgin mosquitoes [31]. Mated mosquitoes are known to carry the hormone, 20-hydroxyecdysone (20E). This hormone has been observed to make the female mosquito more susceptible to infection [31]. The difference in infection observed in these results is likely a reflection of the difference in 20E between the egg group and the virgin group. Future work might explore how differences in 20E between mated mosquitoes affects the number of oocysts produced.

Exflagellation and infection results. The results demonstrate that there is a relationship between exflagellation and infection. As exflagellation increases, oocyst output increases in both groups. As exflagellation increases, infectivity increases logarithmically in both the egg group (R^2 = 0.3903) and the virgin group (R^2 = 0.4294). While these results demonstrate that exflagellation is a variable with influence over the strength of infection produced, exflagellation is unlikely to be the only variable as indicated by the weakness of the R^2. Unfortunately, several obstacles make it difficult to assess exactly how much control exflagellation has over infection.

The primary obstacle is that exflagellation can vary, depending on the person taking the measurement. As discussed previously, an exflagellation measurement cannot be easily replicated by different people. This issue makes it difficult to accumulate experiments showing the effect of exflagellation on oocyst output under different conditions. Under the current method for counting exflagellation, all of the experiments in such a data set would have to be conducted by the same person. Even then, that person's technique is increasingly prone to error over time.

Another obstacle to characterizing this relationship is the difficulty of producing multiple experiments with the same exflagellation. Because exflagellation is a continuous value, the likelihood that two independent experiments in a data set have the same exflagellation is low, and the likelihood decreases as the precision of the measurement increases. It becomes difficult to measure the range of possible infection results from one exflagellation measurement when there are not any independent experiments with the same exflagellation. Given that biological systems are usually stochastic, we should expect some range of outputs from a single input. By comparing the error in the trendline to this hypothetical error attributed to exflagellation, we could determine whether or not there is another variable in the system.

The obstacle that was just described is actually a subset of the first obstacle. While it might be difficult to produce several experiments with the same exflagellation, it is easier to do so if several people can generate exflagellation data for the same data set. This underscores the need for a new measurement method.

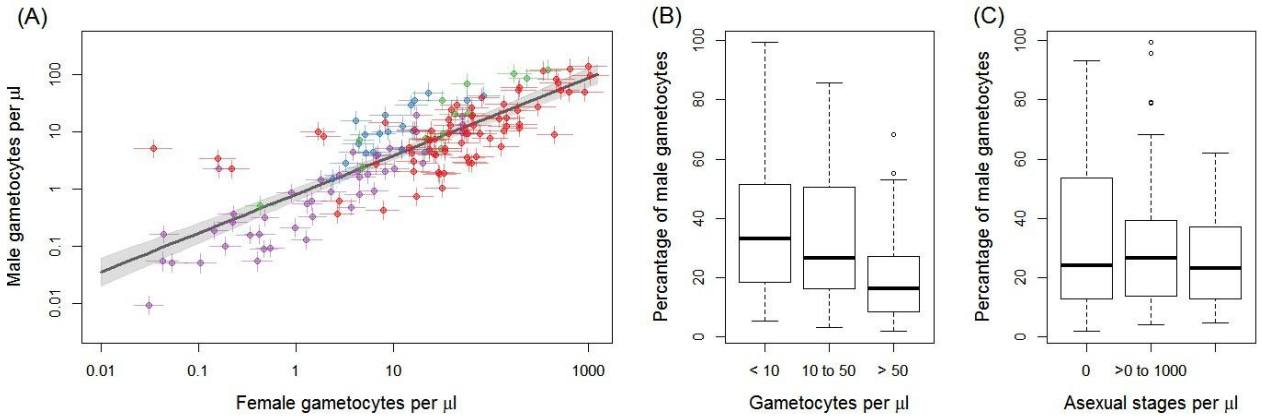

Figure 15. Previous work has shown that gametocyte sex ratio in *P*. falciparum heavily skews female. This figure reports this sex ratio from volunteerswho were carrying *Pf* gametocytes. The samples in **sub-figure A** are from volunteers carrying gametocytes from Ouelessebougou, Mali (red), Yaoundé, Cameroon (green), Bobo-Dioulasso, Burkina Faso (blue) and Balonghin, Burkina Faso (purple) [26].

Sex ratio and infection results. As the ratio of male gametocytes to females approaches 1:1, infection increases (**figures 8, 10**). While this result suggests a significant relationship between sex ratio and infection, the data set has a few issues which limit confidence in the results.

First, there are only 9 experiments that report sex ratio data. The potential for how sex ratio might affect infection was only considered towards the end of the research thesis, so earlier experiments could not be included in the results. The insights provided by the results will be weak, without many experiments to substantiate the relationship. Still, this first set of results suggests that there is a relationship to explore, so more data should be collected to investigate this potential input.

Another limitation is the narrow range of sex ratio values. Most of the recorded sex ratios fall with 0.2 and 0.8 (male/female). This narrow range is actually a feature of *P*. falciparum. The parasite produces significantly more female gametocytes than it produces males [26]. Furthermore, previous work (see **figure 15**) has found that this ratio skews more female as the population of gametocytes in the culture increases [26]. The narrow range of likely sex ratio values suggests that oocyst output should approach some maximum as sex ratio approaches its optimal value within this 0.2 to 0.8 range. While it might be possible to define a strong model within one or two standard deviations of the most frequent sex ratios, such a model would not be good at predicting how infection changes when sex ratio falls outside this range. Ultimately, more data needs to be gathered to assess these possibilities.

Variation in growth medium. Finally, we must acknowledge that the growth medium for the gametocytes contains an enormous amount of variability. Human blood and serum varies greatly between individuals and over time. As discussed in the research methodology, the growth medium was minimally tested against a control group to ensure that it would support gametocyte development. The models produced in this research do not consider any of the specific contents of this growth medium beyond this benchmark test. The differences in, for example, metabolite concentrations within the growth medium might greatly affect the development of gametocytes or change the potency of the gametocytes at maturity. The blood and serum should be characterized as well. This analysis would reveal differences in metabolites between batches of blood and serum. With insights from this characterization we could determine which metabolites should be considered as inputs to the production process. We could also perform more

thorough quality checks against the components utilized in the growth medium for future batches.

Conclusion

The R^2 values of the models described in this paper are not high enough (> 0.98) to consider the relationship between input and outputs wholly predictive. Still, there are several opportunities for development which could eventually yield a deterministic model.

Consider more inputs. The sporozoite production process contains many sources of variation, many of which have not been considered for the models in this paper. While this research has confirmed exflagellation, gametocytemia, sex ratio as considerations for a future deterministic model, a few others are worth including in future work. Blood and serum introduce significant variation to the gametocyte development process. This variation should be captured for any future deterministic model. We can achieve this through a metabolomic study of the growth medium before it is seeded with gametocytes. The metabolomic study would reveal how changes in metabolite concentrations affect the growth of gametocytes and the oocyst output at the end of the process.

Standardize exflagellation and gametocytemia measurements. Enormous progress has been made by previous work which standardized methodology for measuring exflagellation [27]. These protocols allowed the community to agree on a definition for percent exflagellation. However, measurement techniques can still be improved by minimizing the influence of the experiment conductor on the experiment. One solution is to standardize counting methods across labs; however, this is very difficult to achieve and is still vulnerable to human error. A much more elegant solution would automate the counting process. If automated, the margin of error around each exflagellation measurement would decrease considerably. This step would also allow other researchers to easily replicate exflagellation measurements on any sample. Computer vision would be a fantastic technology to help automate exflagellation counting. By taking images of the hemocytometer on a second or millisecond time frame as exflagellation occurs, the images could be animated. A program could be trained to identify exflagellation events by the peculiar movement of the cells. This development would also make exflagellation measurements insensitive to time because the entire reaction could be recorded and replayed. Such a system would also be easy to audit or verify by third parties. It would also provide data on how exflagellation in a culture changes over time. A similar system has already been developed to detect a specific movement pattern in sporozoites. This tool, called ToAST (Tool for Automated Sporozoite Tracking) [14], tracks frame by frame differences across a set of binary images to identify sporozoites gliding in a counterclockwise or clockwise pattern. This tool is open source and could be adapted for exflagellation detection.

A similar approach can be applied for more accurate gametocytemia measurements. Automating gametocyte counting with machine learning would require processing images of sample slides. A generative adversarial network (GAN) framework could be applied to differentiate stage V gametocytes from other gametocytes and cells in a sample. A GAN framework is composed of a generator and a discriminator. The generator produces fake data from the training set while the discriminator attempts to determine which data is real and which is fake. These programs compete with each other over many cycles to produce increasingly convincing fakes. A GAN could be used to "classify" gametocytes in a sample. The GAN could also be trained to differentiate male gametocytes from females, producing a sex ratio for each sample. Given the abundance of images of gametocytes among other cell types, there are plenty of images to train such a network. The greatest challenge will likely be accumulating and labelling these resources to produce a training set.

Changes to experimental setup. We might also consider making changes to the experimental setup for SMFA. The results reflected a large range in oocyst output from mosquitoes of the same batch. The high standard deviation on infection tells us that infection was not distributed evenly. This is supported by the results, where so few mosquitoes developed incredibly high infection while the majority developed weak or no infection. This distribution suggests that the first few mosquitoes to feed are consuming a much higher parasite load than that consumed by the majority of the batch. We can reduce the variance of infection in a batch by ensuring that the infected blood is well mixed as the mosquitoes consume it. This change would ensure that the first mosquitoes do not receive a significantly higher concentration of gametocytes than that received by the rest of the batch.

Another interesting idea is to increase infectivity in the batch by somehow doping the mosquitoes with the hormone 20E. This hormone seems to make egg-carrying mosquitoes more susceptible to infection than virgin mosquitoes are [31]. Future work could explore what optimal concentration of this hormone maximizes sporozoite yield from mosquito batches. However, we would need to find a way for all mosquitoes in the batch to express or sustain this hormone as infection takes hold.

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Supplementary Information

See this Github repository for the raw data and data exploration which informed this paper. See **tables 1** and **2** on the next page for a summary of the raw data and standard deviations of oocyst count.

Table 1. *Virgin mosquitoes.* A summary of experimental data obtained between Feed 15-20.

Culture	Strain / Code	Feed	Exflagellation (%)	Oocysts, avg. per mq	Oocysts, std. dev.	Infectivity (%)
A	NF135	15	0.0322	0.00	0.0000	0.00
B	NF54	15	0.0322	0.25	0.4600	25.00
A	NF54 / T9	16	0.4970	10.83	7.4200	83.33
B	NF54 / T7	16	0.4074	11.63	8.6300	75.00
C	NF54 / FARAH	16	0.2369	14.18	9.7300	90.91
A	NF54	17	0.2031	0.00	0.0000	0.00
B	NF135	17	0.0280	0.00	0.0000	0.00
C	APL5G / NICK	17	0.2226	0.09	0.3000	9.09
A	NF54 / T4	18a	0.0712	0.58	1.2400	25.00
B	NF54 / T7	18a	0.0471	0.26	0.4500	26.32
C	NF54 / T9	18a	0.0515	0.69	1.3800	23.08
A	NF54 / T9	18b	0.2327	3.09	4.5700	36.36
A	NF54 / T7	19	0.3378	0.30	0.6700	20.00
B	APL5G / NICK	19	0.1974	0.33	0.4900	33.33
C	G8	19	0.2121	19.50	8.5300	100.00
A	NF54 / T7	20	0.6127	4.07	7.7524	70.00
B	APL5G / NICK	20	0.5164	3.40	7.3596	54.55
C	G8	20	1.0090	14.86	14.3827	77.78

Table 2. *Egg-carrying mosquitoes.* A summary of experimental data obtained between Feed 15-20.

Culture	Strain / Code	Feed	Exflagellation (%)	Oocysts, avg. per mq	Oocysts, std. dev.	Infectivity (%)
A	NF135	15	0.1298	0.57	0.7900	42.86
B	NF54	15	0.1298	0.07	0.2600	6.67
A	NF54 / T9	16	0.4970	14.20	11.9200	80.00
B	NF54 / T7	16	0.4074	9.80	8.9800	80.00
C	NF54 / FARAH	16	0.2369	16.50	13.9600	75.00
A	NF54	17	0.2031	0.00	0.0000	0.00
B	NF135	17	0.0280	0.00	0.0000	0.00
C	APL5G / NICK	17	0.2226	0.00	0.0000	0.00
A	NF54 / T4	18a	0.0712	1.60	2.3000	40.00
B	NF54 / T7	18a	0.0471	0.77	1.4200	38.46
A	NF54 / T9	18b	0.2327	12.36	7.5200	100.00
A	NF54 / T7	19	0.3378	1.50	1.9700	50.00
B	APL5G / NICK	19	0.1974	0.67	0.8200	50.00
C	G8	19	0.2121	20.20	13.2700	100.00
A	NF54 / T7	20	0.6127	12.80	7.6616	100.00
B	APL5G / NICK	20	0.5164	5.89	4.2262	88.89
C	G8	20	1.0090	38.60	8.9331	100.00

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