Trends in Parasitology

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REVIEWS

Review

When Is a *Plasmodium*-Infected Mosquito an Infectious Mosquito?

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Plasmodium **parasites experience significant bottlenecks as they transit through the mosquito and are transmitted to their mammalian host. Oocyst prevalence on mosquito midguts and sporozoite prevalence in salivary glands are nevertheless commonly used to confirm successful malaria transmission, assuming that these are reliable indicators of the mosquito's capacity to give rise to secondary infections. Here we discuss recent insights in sporogonic development and transmission bottlenecks for *Plasmodium*. We highlight critical gaps in our knowledge and frame their importance in understanding the human and mosquito reservoirs of infection. A better understanding of the events that lead to successful inoculation of infectious sporozoites by mosquitoes is critical to designing effective interventions to shrink the malaria map.**

Highlights

The discrepancy between exposure to infected mosquito bites and malaria incidence suggests a transmission bottleneck that is currently understudied.

Recent studies from non-human malaria models are indicative of a minimum salivary gland sporozoite density that is required to achieve infection following a mosquito bite.

Infection-induced alterations in blood-feeding behavior of mosquitoes may influence natural transmission dynamics.

The Rise of Sporozoites

Malaria, the deadliest human vector-borne disease, is caused by parasites of the genus *Plasmodium* and is transmitted by *Anopheles* mosquitoes. Transmission of *Plasmodium* parasites between their mosquito and mammalian hosts is a bottleneck for the parasite and constitutes vulnerabilities that could be leveraged in malaria-elimination efforts (Figure 1). Transmission from humans to mosquitoes starts with sexual commitment following activation of Apatella2-g (*PfAP2-G*) [1,2], that is under epigenetic control of heterochromatin protein 1 (PfHP1) [3] and gametocyte development 1 (GDV1) [4], leading to the development of male and female gametocytes within the human host [5–7]. Gametocytes sequester away from the circulation during their 8–12 day maturation [8,9], and circulate once released for an average of 2.7–6.4 days in the bloodstream [9–11] to be taken up by blood-feeding mosquitoes. Upon ingestion by mosquitoes, a single activated female gametocyte becomes one macrogamete whilst a single male gametocyte gives rise to eight motile microgametes [12,13] that locate and fertilize female macrogametes to form diploid **zygotes** (see Glossary) [14–16]. Zygotes transform into ookinetes that penetrate the mosquito midgut to form oocysts on the basal side of the midgut [12]. Multiple rounds of genomic DNA replication results in a multinucleated cell, a syncytium, where thousands of daughter cells known as sporozoites are formed after synchronized budding from the sporoblast bodies of the cell [15]. Mature sporozoites exit the oocyst into the open circulatory system of the mosquito with a proportion successfully invading the mosquito salivary glands [17,18]. Sporozoites remain in the salivary glands and may render adult mosquitoes infectious for the remainder of their lifespan [16]. The rate at which sporozoites are inoculated into the next host, how this is related to the density of gametocytes in the human host, oocyst burden and salivary gland sporozoite burden are matters of current debate [19–21]. It is, however, evident that sporogonic development in mosquitoes involves several bottlenecks where *Plasmodium* parasites are present in vulnerably low numbers (Figure 1). Here, we review the available literature on developmental bottlenecks in mosquitoes with a focus on recent manuscripts on the transition from salivary gland sporozoites to skin sporozoites. We argue that this understudied area of malaria transmission is of key importance to better appreciate the dynamics of malaria transmission in natural settings, quantify the contribution of different populations of infected individuals

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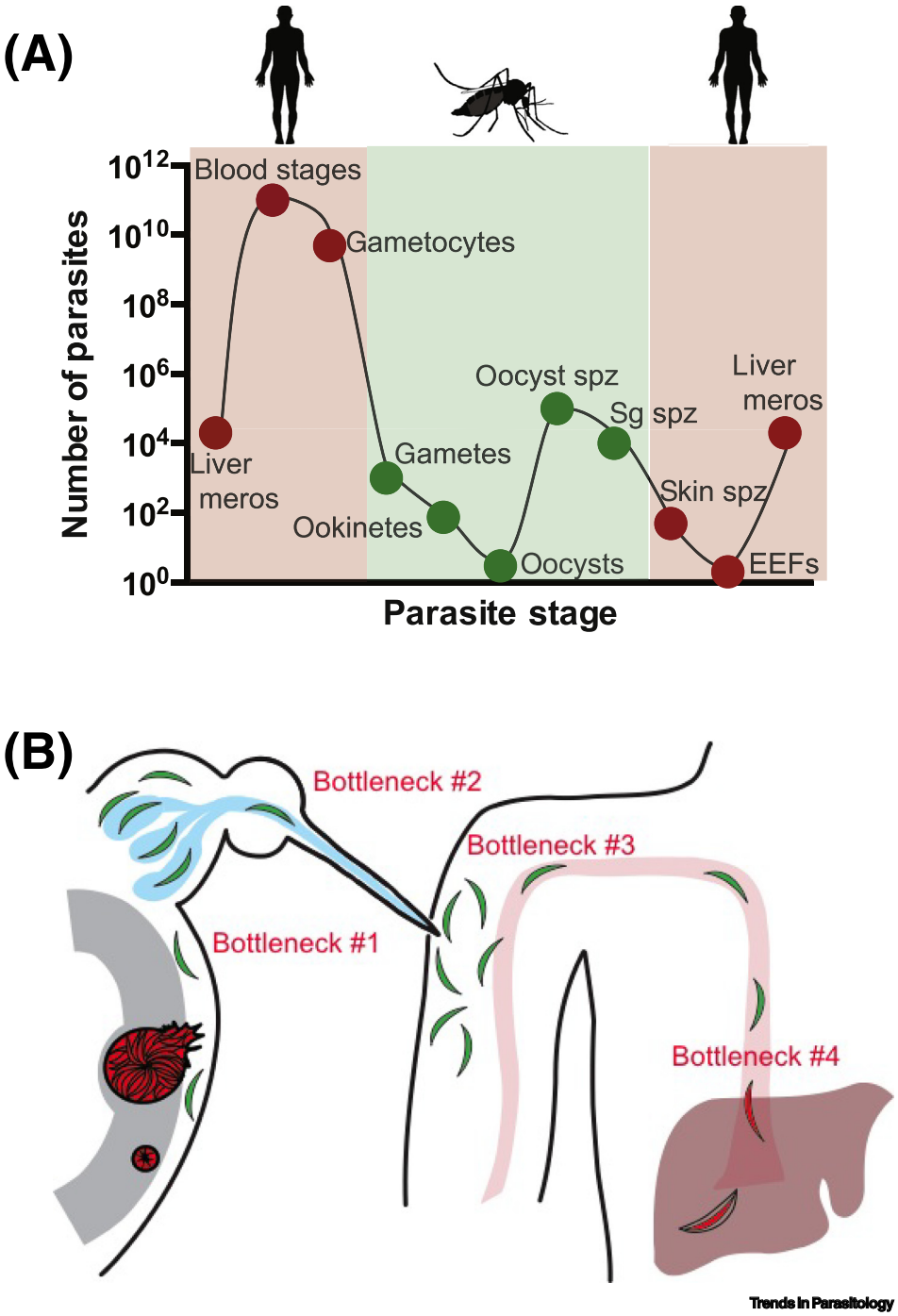


Glossary

Anterior: used to describe anatomy, meaning towards the head; opposite of posterior.
Circumsporozoite protein (CSP): the most abundant protein present in the oocyst/sporozoite stage from day 7 postinfection onwards.
Distal: located away from an area, further away from the center; opposite of proximal.
Zygote: a diploid cell formed by fusion of haploid gametes becoming a fertilized ovum.

Figure 1. Transmission Bottlenecks in the *Plasmodium* Life Cycle. (A) Estimated parasite numbers during the different life-cycle stages reveals that transmission to and from the mosquito is associated with significant decreases in parasite numbers (meros, merozoites; spz, sporozoites; sg salivary gland; EEFs, exoerythrocytic forms). Adapted from Povelones *et al.* [109]. (B) Specific bottlenecks faced by *Plasmodium* sporozoites: cartoon of the sporozoites' journey from mosquito to mammalian host, highlighting the following bottlenecks: #1 estimates suggest ~20% of oocyst sporozoites reach the salivary glands; #2 less than 1% of salivary gland sporozoites are expelled during probing; #3 ~20% of inoculated sporozoites enter the bloodstream; #4 represents several unmeasured bottlenecks, namely the efficiency with which sporozoites arrest in the liver, enter hepatocytes, and develop into liver stages. Artwork by Brandy Lee Bennett.

(e.g. high- and low-density gametocyte carriers) to onward transmission and predict the impact of malaria interventions.

Sporozoite Development in and Egress from Oocysts

Oocysts are typically detected by microscopy on the *Anopheles* mosquito's midgut wall 7–9 days after an infectious bloodmeal, although some markers allow much earlier oocyst detection [22]. The density of oocysts is strongly determined by the gametocyte density in the peripheral blood of the infectious human host that formed the source of the mosquito's infection [23,24]. Following the formation of oocysts on the mosquito's midgut basal wall, a massive expansion of parasite numbers occurs through a replication process known as schizogony. This is a syncytial mode of replication in which genomic DNA replication, after multiple rounds of mitotic nuclear division, precedes cytoplasmic compartmentalization into individual sporozoites by the formation of cytoplasmic islands [15], called sporoblasts, from which sporozoites bud, each containing one nucleus and the appropriate number of individual organelles, ultimately filling the oocyst with thousands of crescent-shaped sporozoites (10–15 μm by 1 μm in diameter). One successful oocyst produces between 1500 and 5000 individual sporozoites [25,26]. Sporozoite egress is required for sporozoite release in the mosquito's hemocoel and is actively established by parasite-dependent proteolysis. In this process, reviewed by Kojin *et al.* [27], a parasite-derived cysteine protease plays a central role in rupture of the capsule [28] – together with the **circumsporozoite protein** (**CSP**), which can be found on the oocyst plasma membrane and the inner surface of the capsule [29]. Recently two essential proteins were identified, oocyst rupture protein (ORP) 1 and 2, that promote heterodimer formation in the oocyst after maturation, possibly leading directly or indirectly to destabilization and the activation of the cysteine protease [30,31]. Since rupture occurs for the majority of oocysts in low-infected mosquitoes [21], it is generally assumed that oocyst positivity is a reliable indicator of later infectivity of mosquitoes. This, however, depends on the migration of sporozoites into salivary glands.

Sporozoite Migration into Salivary Glands

Upon exit from the oocyst, sporozoites enter the open circulatory system of the mosquito, which consists of a dorsal vessel, spanning the length of the mosquito, that contracts to generate waves of directional flow. Released sporozoites can enter the abdominal portion of the dorsal vessel through openings, called ostia, and be passively carried **anteriorly** with the flow, exiting the vessel in the thoracic cavity near the salivary glands [32–34]. Sporozoites that do not enter the dorsal vessel are carried with the flow of hemolymph throughout the mosquito body, and while a proportion likely enter salivary glands, many can be found trapped in the mosquito's appendages, the alary muscle, and other locations [33,35]. The hemolymph contains immune factors and phagocytic cells, called hemocytes, and one study found evidence of sporozoite degradation in the hemolymph [33]. Nonetheless, the degree to which this occurs and the mechanism(s) by which this occurs remain understudied. There is a paucity of studies that have looked at the efficiency with which oocyst sporozoites colonize salivary glands [26,33]. Rosenberg *et al*. counted the sporozoites in single oocysts of *Plasmodium falciparum*- and *Plasmodium vivax*-infected mosquitoes and compared these numbers with a previous study in which salivary-gland sporozoites were enumerated; they estimated that 20% of oocyst sporozoites successfully enter salivary glands [26]. In another study, using mosquitoes heavily infected with the rodent malaria parasite *Plasmodium berghei*, a 10-fold lower efficiency of salivary gland entry by sporozoites was estimated [18].

Upon arrival at the glands, entry is dependent on recognition events between sporozoites and salivary-gland proteins, a process reviewed in detail by Ghosh and Jacobs-Lorena [36], Mueller *et al*. [17], and Kojin *et al.* [27]. On the sporozoite side, CSP, thrombospondin-related anonymous

protein (TRAP), TRAP-related protein (TREP), and apical membrane antigen/erythrocyte binding-like protein (MAEBL) have been shown to be involved in this process [37,38]. CSP is known to bind to heparan sulfate proteoglycans and this may constitute the basis for the initial recognition event as these glycans are found on salivary glands [39,40]. On the host side, several salivary-gland proteins involved in sporozoite invasion have been identified – CSP-binding protein (CSPBP), salivary gland surface protein 1 (SGS1), and Saglin being the best characterized [41,42]. CSPBP and Saglin were identified in screens using CSP or TRAP, respectively. CSPBP- or SGS1-specific antibodies and peptides inhibiting the TRAP–Saglin interaction decrease invasion of the salivary glands by sporozoites. However, the specific role of Saglin, as an essential salivary-gland receptor for sporozoite invasion, was recently doubted when no protein expression was observed in the **distal** lateral lobes of the salivary gland, a primary sporozoite invasion site [43]. Together, these studies suggest that entry into salivary glands is a complex process involving several sporozoite proteins as well as mosquito glycans and proteins. Indeed, visualization of this process by detailed, sequential electron micrographs suggests that there is an initial recognition event between the sporozoite surface coat and the salivary gland basal lamina, followed by tighter adhesion and entry into the cells [44]. Additional binding studies, as well as *in vivo* knockdown studies in the mosquito using RNAi, are needed to further elucidate the molecular events involved in this process [45,46]. Experiments that directly examined the number of ruptured oocysts in relation to salivary gland sporozoite load suggest that, on average, 1250 sporozoites reach the salivary gland per ruptured oocyst [21]; other studies estimate higher sporozoite numbers per oocyst but do not directly relate this to oocyst rupture [25,26]. Whilst these numbers are deemed sufficient to render a mosquito infectious for the remainder of her life, there are data suggesting that infectiousness decreases as sporozoites age, further complicating assessments of a mosquito's infectious potential in the field [47,48].

Sporozoite Residence in Salivary Glands

The salivary glands of female mosquitoes are paired organs, one on each side of the esophagus, with each gland consisting of three lobes, two lateral lobes, and a shorter median lobe. Each lobe is organized as a single layer of cup-shaped epithelial cells surrounding a large secretory cavity and a central salivary canal. In the distal portion of the glands, the salivary duct is continuous with the secretory cavity; however, as one moves anteriorly towards the proboscis, the ducts narrow, to about 1 μm in diameter, which is slightly wider than a single sporozoite, and become chitinized, eventually joining with the duct from the opposite gland to form the common salivary duct [49]. Several studies have found that sporozoites preferentially enter the distal portions of the lateral and median lobes where the ducts are continuous with the secretory cavity [45,50]. After their entry, sporozoites move into the secretory cavity and a few can be found in the salivary duct, awaiting their inoculation into the mammalian host [44]. Sporozoites that enter the more proximal portions of the gland appear to be 'landlocked' and may not be able to enter the salivary canal [51]. Thus, the process of localization to the salivary glands and entry into the secretory cavities of the glands presents many barriers to transmission [52].

Mosquito Blood-Feeding Physiology and Sporozoite Inoculation

Plasmodium parasites take advantage of the obligate blood-feeding behavior of the mosquito to enter their mammalian host. After the mosquito stylet pierces the skin it commences to search for blood, the labrum thrusting and bending to survey the entire area within its reach. Release of saliva occurs during probing [53], contributing to the mosquito's ability to find blood due to saliva proteins that counteract the hemostatic and inflammatory responses of the host. The probing phase ends when the mosquito locates blood, having cannulated a vessel or created a hematoma from the rupture of capillaries. There is no evidence of significant salivation during imbibement of blood [49,53], though, if it does occur, the difference in the flow rate of saliva

compared with the counter flow of blood into the mosquito, estimated to be 10^4–10^5 times faster, would result in reingestion of the saliva secreted during blood feeding. Indeed, sporozoites have been found in the midguts of blood-fed mosquitoes [54,55]. The physiology of blood feeding suggests that sporozoites are predominately inoculated into the extravascular tissue. Experiments in which the bite site was removed, transplanted to naïve animals, or artificially heated, and *in vivo* visualization of the process of sporozoite inoculation, all support the notion that the majority of sporozoites are inoculated into the skin [56–61]. This is further supported by a recent study demonstrating that bloodmeal acquisition is not associated with a higher rate of infection [62]. Due to the length of the mosquito's stylet the majority of sporozoites are inoculated into the dermis and a small proportion into the epidermis or subcutaneous tissue [63]. To continue their life cycle, sporozoites need to travel great distances compared with other morphological stages. This is achieved by translocating actively, by gliding motility and cell traversal, or passively, after entering vessels of the bloodstream or lymphatic system. In the dermis they actively glide forward using the actin/myosin-based motor [60,61,64] to enter vessels of the bloodstream. Only those that enter the bloodstream, and not those drained in the lymphatic system, can give rise to human infection [61,64].

Inoculum Size and Its Relation to Mosquito Salivary Gland Load

Sporozoite entry into the host is a critical time for both host and parasite, with factors such as inoculum size likely important in determining whether the pathogen succeeds in establishing a foothold. Different approaches have been used to estimate the inoculum: initial studies induced infected mosquitoes to salivate [65–69], counting the ejected sporozoites, and more recent studies utilized the rodent malaria model [59,70], allowing mosquitoes to probe on an anesthetized mouse and quantifying inoculated sporozoites by PCR or microscopy. Though these studies differed in their methodologies, some common features emerged: (i) in all cases, mosquitoes inoculated only a small proportion of the sporozoites in their salivary glands, generally less than 1%; (ii) the majority of mosquitoes ejected few sporozoites, with median inocula ranging between 8 and 39 sporozoites. A minority of mosquitoes ejected >100 sporozoites, the percentage of these high-injectors ranging from 7 to 36% in the different studies. The large range of high injectors may be due to differences in experimental set-up or the distribution of salivary gland sporozoite loads in mosquitoes. Murine models with non-human *Plasmodium* species typically give higher estimates of expelled sporozoites, with 28–50% of mosquitoes inoculating >100 sporozoites, while such large inocula are less frequently observed in mosquito spitting experiments with human malaria parasites. It is possible that this difference reflects a biological difference between mosquitoes infected with rodent parasites and *P. falciparum* sporozoites. Alternatively, it could result from differences in protocols, with rodent work being performed with live animals that provide more natural biting circumstances, compared with studies that induced infected mosquitoes to salivate, and may thus better simulate salivation and sporozoite mobilization and ejection. Differences in total sporozoite load in salivary glands also form a plausible factor for a higher inoculum size in murine models: rodent malaria-infected mosquitoes frequently achieve high infection burdens. Though these studies did not find a strong correlation between the size of the sporozoite inoculum and sporozoite density in the glands, the number of infected mosquitoes analyzed in each study may have been too small to detect differences against the backdrop of high biological variability [59,71]. Considering the large variation in sporozoite densities observed in mosquito populations, understanding the association between sporozoite load, inoculum size, and the likelihood that this inoculum gives rise to secondary infections is of crucial importance to accurately quantify the contribution of different hosts to transmission. The contribution of human hosts to transmission is dependent on mosquito biting behavior. Due to their low reserves, anophelines frequently seek more than one bloodmeal during a single gonotrophic cycle. Frequent bloodmeals may not only accelerate sporozoite development [72], this central aspect of

anopheline behavior may also favor parasite transmission with the entomologic inoculation rate (EIR) potentially increasing by a factor equal to the number of bites per gonotrophic cycle [73]. Intriguingly, evidence is accumulating that pathogen–vector manipulation may further enhance transmission (Box 1).

The Likelihood That a Single Infected Mosquito Bite Will Result in a Malaria Infection

The EIR is a quantification of malaria exposure that is central to malaria epidemiology. EIR is defined as the number of infected mosquito bites per person per time-unit; it is estimated based on mosquito density, mosquito biting frequency, and the proportion of mosquitoes with sporozoites in their salivary glands [74]. EIR does not explicitly consider how heavily infected mosquitoes are or the likelihood that an infected mosquito bite will result in a blood-stage infection [75]. Early malariologists noted a significant discrepancy between human exposure to infected mosquitoes and the incidence of malaria infection [76–79], suggesting that the majority of infected bites may not result in a detectable infection. Attempts to indirectly determine the proportion of infected mosquito bites that result in malaria infection, measuring infant infection rates and mosquito biting rates, estimate that between 1 and 10% of infective bites lead to infection [77]. More recently, a rodent model was utilized to directly quantify infection probability after a single infected mosquito bite and found that 17% of infected bites resulted in a blood-stage malaria infection [62]. These data suggest that the majority of infected mosquito bites may not result in a blood-stage infection. Whilst human and epidemiological factors also play important roles in determining the relationship between EIR and (clinical) malaria incidence (Box 2), the discrepancy between both estimates suggests that there may be a relevant transmission bottleneck involving sporozoite expelling and downstream barriers in the mammalian host. Does sporozoite density in the salivary glands play a role in infection probability? The few studies addressing this topic, predominantly relying on *Plasmodium yoelii* and *P. berghei*, reported that bites from mosquitoes with higher sporozoite loads were more likely to initiate infection [19,62,80]. Modeling vaccine trial data from both rodent and human studies, Churcher *et al.* found that mosquitoes with >1000 *P. berghei* sporozoites initiate infection 78% of the time with considerably lower likelihood of infection at lower sporozoite densities [19]. Using the rodent *P. yoelii* malaria model, Aleshnick *et al.* found that a threshold model best describes the data, with a jump in infection likelihood occurring at sporozoite densities of ~10 000. Once this threshold was met, infection probability plateaued at ~40% [62]. Whilst both studies found an association between sporozoite salivary gland load and the likelihood of onward infection, the study by Churcher *et al.* predicted much higher rates of infection by single infected mosquitoes

Box 1. Does *Plasmodium* Manipulate Its Mosquito Host to Increase Transmission Likelihood?

There is a growing body of work investigating the influence of *Plasmodium* infection on mosquito blood-feeding behavior. A recent detailed study on mosquito bloodmeal preference observed that, across the transmission season and dry season, ~20% of mosquitoes may take multiple human bloodmeals during a single night [90]. These repeated, partial bloodmeals may be associated with the mosquito infection status. Behavioral studies using field-caught *P. falciparum*-infected and -uninfected *Anopheles gambiae* and *Anopheles funestus* found that a higher percentage of sporozoite-infected mosquitoes initiated probing and that they probed longer than uninfected mosquitoes [91]. Additionally, field-caught infected anophelines were more likely to have evidence of ≥2 bloodmeals compared with uninfected mosquitoes [92,93]. This is supported by genotyping of blood-stage parasites from members of the same household in which it was found that a much higher frequency of identical genotypes was found in household members than expected [94], suggestive of inoculations by the same mosquito into multiple human hosts. Furthermore, a laboratory model using rodent *Plasmodium yoelii* and *Anopheles stephensi* mosquitoes showed increased biting and number of probes by sporozoite-infected mosquitoes compared with uninfected mosquitoes [95]. One possible mechanism underlying these changes in feeding behavior is suggested by data demonstrating that salivary gland sporozoites decrease the level of apyrase in the mosquito host's saliva, making it more difficult for them to feed to repletion on one host [96,97]. It is predicted that such changes in feeding behavior would significantly increase the likelihood that a single infected mosquito transmits sporozoites [98,99].

Box 2. What Explains the Low Transmission Efficiency of Malaria?

The entomologic inoculation rate (EIR) is the product of the number of mosquito bites experienced by humans per unit time and the proportion of these mosquitoes that is sporozoite-positive. Not all infectious bites successfully achieve infection in human hosts. Transmission efficiency is defined as the number of infections in humans that is achieved per infectious bite and can be estimated by the number of infections acquired by humans per unit time (the force of infection; FOI) relative to EIR [100]. The observation that malaria incidence is often lower than expected based on EIR may plausibly be related to the fact that not all sporozoite-positive mosquitoes are infectious. There are alternative explanators for low transmission efficiency. Transmission efficiency decreases with increasing EIR [100]: in areas of higher malaria transmission intensity, where human populations are more heavily exposed, a smaller fraction of all infectious bites results in blood-stage malaria. This suggests that epidemiological characteristics and host factors may be at play. The fact that not all incident infections result in clinical symptoms (or in treatment-seeking behavior in case of passively collected data) will result in some infectious bites not contributing to measured clinical incidence. However, also in carefully monitored cohorts with regular active screening for (asymptomatic) infections, the number of incident infections is typically much lower than expected based on EIR estimates [79,100–102]. Naturally acquired immune responses are unlikely to prevent sporozoite inoculations from achieving blood-stage infection [103,104], although effective blood-stage immunity may suppress parasite densities to levels undetectable by microscopy [103,105] and thus result in incident infections going unnoticed. Another important factor in understanding transmission efficiency is variation in exposure that is experienced by individuals living in endemic areas. This so-called heterogeneity in malaria exposure in space and time is a major determinant of malaria incidence patterns in populations [102,106]. Whilst heterogeneous mosquito exposure can amplify transmission of pathogens if a minority of heavily infected individuals infects many mosquitoes [107], inoculations on the same host may also dampen transmission efficiency [108]. If infectious bites are disproportionally experienced by a subset of the population, especially if this exposure occurs over a short time period of intense exposure, many sporozoite inoculations may result in superinfections but will not be detected as incident infections. These sporozoite inoculations in individuals who are already infected thus contribute to the disconnect between EIR and FOI estimates. A possible impact of inefficient expelling of sporozoites by mosquitoes with low salivary gland sporozoite loads will need to be quantified in the context of these other determinants of transmission efficiency.

with lower sporozoite burdens. The protocol used in controlled human malaria infection (CHMI) trials, a model that is increasingly used to study sporozoite infectivity and evaluate new vaccines or drugs [81], may help to explain this discrepancy. Naïve volunteers are typically exposed to a minimum number of five bites of blood-fed mosquitoes to ensure homogeneous exposure between volunteers and infection in all control subjects. Analysis of 47 individuals participating in CHMI found that only mosquitoes with >1000 *P. falciparum* sporozoites remaining in the salivary gland after blood-feeding were capable of inducing an infection in humans [19]. By contrast, review of data from 13 CHMI studies with a total of 75 volunteers found no correlation between the time to parasitemia or height of first parasitemia, a readout indicative of liver load [82], and mean sporozoite load in mosquitoes [20]. This discrepancy may be explained by the fact that all mosquitoes in the studies analyzed by Walk *et al*. were heavily infected (range 26.500–160.500 *P. falciparum* sporozoites/salivary gland) [20]. In terms of sporozoite inoculum size and the likelihood of infection, the interpretation of CHMI data is complicated by two factors: (i) in general, only highly infected mosquitoes are used and this is not reflective of salivary gland sporozoite densities in naturally infected mosquitoes, and (ii) bloodmeal acquisition is used as a readout for a successful encounter, rather than mosquito probing. Whilst this is understandable from a practical point of view, successful probing being difficult to quantify, it means that CHMI studies do not take into account those mosquitoes that probe but do not take a bloodmeal whilst it is known that these mosquitoes inoculate sporozoites and can initiate infection [56,58,59,62]. This omission of mosquitoes that fail to take a bloodmeal may result in a higher estimated transmission efficiency since some probing mosquitoes (potentially inoculating sporozoites) are excluded. The magnitude and importance of this plausible overestimation of transmission efficiency in CHMI studies are currently unknown.

The Human Infectious Reservoir: The Gap between Oocyst Prevalence and Efficient Sporozoite Inoculation

Extrapolating these findings to natural malaria transmission is not trivial since very limited data exist from human malarias and the estimated minimum salivary gland sporozoite density required for

successful infection differs considerably between studies [19,79]. Sporozoite numbers in wild-caught mosquitoes are predominantly <10 000 sporozoites per infected mosquito, mirroring the oocyst distribution and the proportion of mosquitoes with one or two oocysts (Figure 2) [66,71,72]. If the same threshold sporozoite density observed by Aleshnick *et al.* for *P. yoelli* applies to natural *P. falciparum* infections, this would suggest that the majority of naturally infected mosquitoes may be unlikely to transmit their infection. If one successful ookinete produces between 1500 and 5000 individual sporozoites [25,26] and a proportion of these sporozoites reach the salivary glands, infections with a natural median of one or two oocysts per mosquito would be on the threshold of plausible malaria transmission. Importantly, in xenodiagnostic surveys that aim to quantify the contribution of different populations to the human infectious reservoir for malaria, low oocyst burdens also dominate. High oocyst densities are regularly observed in mosquito-feeding experiments on selected high-density gametocyte carriers [83] but since most natural gametocyte carriers harbor very low gametocyte densities [84] low oocyst densities are typically observed in experiments where blood donors were recruited without prior gametocyte screening [85,86]. In such population-wide assessments of infectivity it is typically observed that only 1–11% of the general population residing in malaria-endemic areas is capable of infecting mosquitoes at the moment of sampling [87]. The majority of these infectious individuals infect only few mosquitoes (34–76% of infectious individuals infect <5% of mosquitoes feeding on their blood sample [85,86,88]). This low infection prevalence is accompanied by a low burden of oocysts in infected mosquitoes [23,83,89]; 31–60% of infectious individuals in recent xenodiagnostic studies in Burkina Faso and Ethiopia infected mosquitoes with one or two oocysts only, not achieving higher oocyst burdens [86,88]. If these infected mosquitoes are indeed on the threshold of plausible transmission this would annul the contribution to transmission of a large proportion of infectious individuals. Better data on sporozoite expelling in relation to gametocyte density, oocyst burden, and salivary gland sporozoite burden are thus urgently needed.

Concluding Remarks

This review summarizes our current understanding of *Plasmodium* parasite bottlenecks as they relate to sporozoite development, migration, and transmission. Sporozoite development in oocysts represents the only expansion of parasite numbers in the mosquito host. Following this, the parasite encounters a series of bottlenecks that reduce its numbers considerably by the time the parasite develops into the next life-cycle stage, the exoerythrocytic schizont in human hepatocytes. In reviewing what is known of these bottlenecks it is clear that there remain large gaps in our knowledge (see Outstanding Questions). The relationship between oocyst burden and salivary gland sporozoite load remains incompletely understood. Since field work estimates of infection often only involve measuring oocyst burden (and commonly express transmission outcomes in terms of the proportion of oocyst-positive mosquitoes), it is of crucial importance to estimate the likelihood of successful sporozoite infection in relation to oocyst density and, if possible, the likelihood of successful hepatocyte infection or subsequent blood-stage infection. Not all of these parameters are easy to obtain for human malarias and some may need to be approximated using non-human malaria models or mathematical models. Current estimates based on *P. berghei* are likely to underestimate the success rate of oocyst sporozoites because this laboratory model gives rise to high numbers of oocysts, many of which never fully develop [18]. Better measurements on mosquitoes with oocyst numbers in the range of wild-caught mosquitoes (one to five oocysts/gut) are necessary if we are to close this knowledge gap. If low oocyst densities are unlikely to result in salivary gland sporozoite loads sufficient for efficient onward transmission, transmission-blocking interventions might not need to prevent all infected mosquitoes as long as high oocyst burdens are prevented. If, on the contrary, low oocyst densities regularly result in high numbers of expelled sporozoites, transmission-blocking intervention would need to completely eliminate mosquito infection.

Outstanding Questions

How diverse are *Anopheles* in terms of innate susceptibility to malaria parasites and blood-feeding physiology?

What is the efficiency with which oocyst sporozoites infect salivary glands? How does oocyst number correlate with salivary gland load?

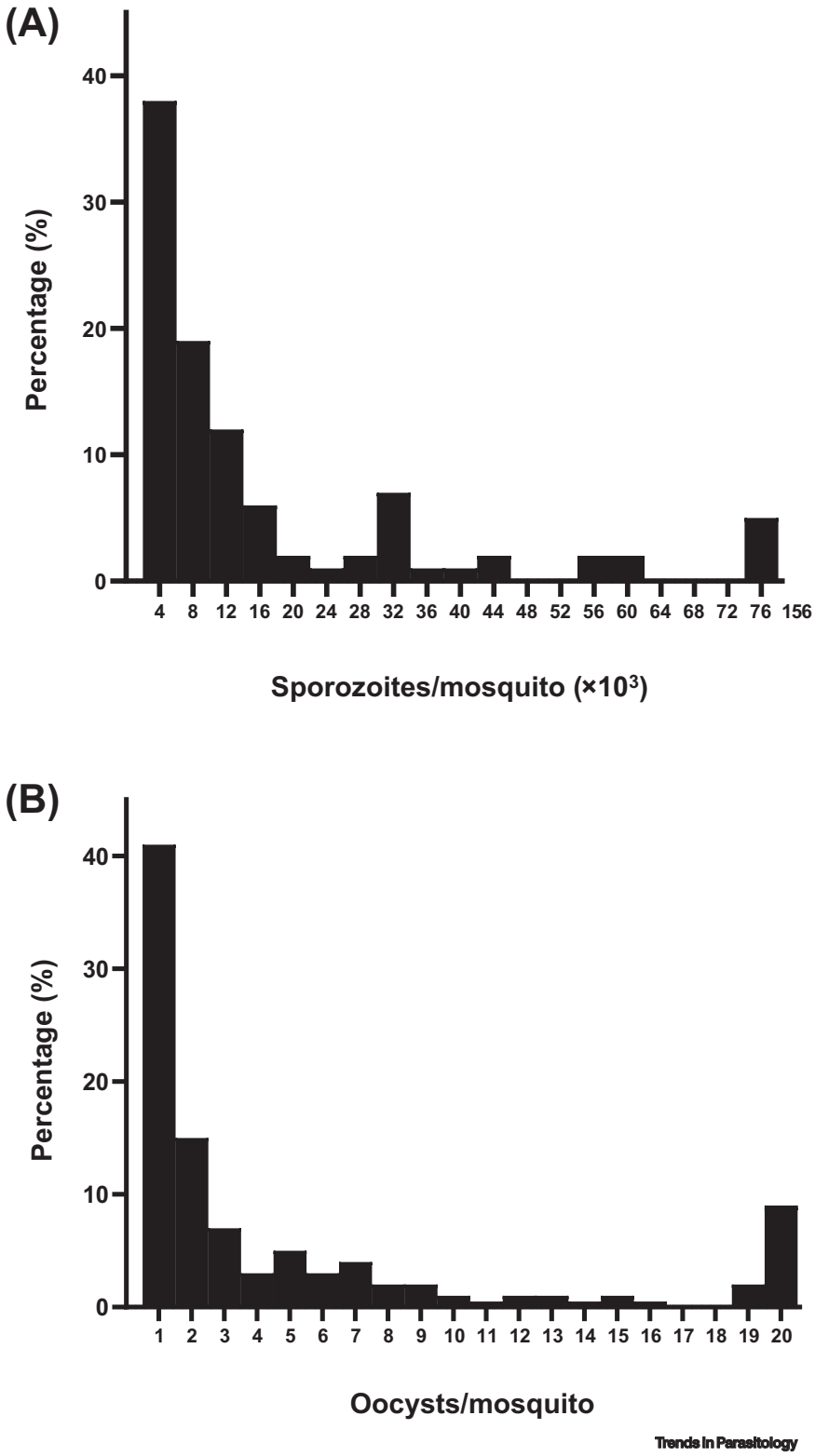
What is the association between sporozoite load and the number of expelled sporozoites in natural infections?

How are sporozoites expelled during repeated probing events, especially if these occur over a short period of time?

What human populations are associated with the most infectious mosquitoes?

To what extent does mosquito feeding or probing on multiple hosts contribute to transmission dynamics?

Though evidence suggests that the majority of sporozoites are inoculated into the dermis, is there a significant minority that are inoculated directly into the bloodstream and, if so, how does this affect transmission efficiency?



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These questions require a better understanding of the quantitative dynamics of the sporozoite's transition from oocyst to salivary gland. Quantifying differences among different *Anopheles–Plasmodium* species combinations found in the endemic areas is crucial to the prediction of these dynamics. In addition, a better understanding is required of infection-induced alterations in the blood-feeding behavior of mosquitoes. These behavioral studies should be expanded to control for mosquito age and to a range of field-relevant infection levels to be meaningful for our understanding of natural transmission.

In conclusion, successful transmission by *Plasmodium*-infected mosquitoes depends on several poorly understood factors related to the mosquito vector. These include the efficiency with which sporozoites travel from the midgut to the salivary glands, the impact of mosquito gland load on infection likelihood, differences among mosquito species in their transmission of sporozoites, and the impact of infection on mosquito behavior. Quantification of these events is critical to understand malaria transmission efficiency and define the minimum efficacy required from transmission-blocking interventions. Understanding these factors will allow the identification of human populations that harbor infections capable of rendering mosquitoes not only infected but truly infectious, thus supporting malaria-elimination efforts.

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Figure 2. Oocyst and Sporozoite Distributions in Wild-Caught Mosquitoes. Frequency distribution of (A) oocyst numbers and (B) salivary gland sporozoite numbers in wild-caught *Plasmodium falciparum*-infected *Anopheles gambiae* mosquitoes (n = 94). Adapted from Collins *et al.* [71].

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