Molecular and ecological factors modulating the fitness of Anopheles gambiae mosquitoes infected with Plasmodium falciparum

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Molecular and ecological factors modulating the fitness of *Anopheles gambiae* mosquitoes infected with *Plasmodium falciparum*

A dissertation presented

by

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Molecular and ecological factors modulating the fitness of *Anopheles gambiae* mosquitoes infected with *Plasmodium falciparum*

**ABSTRACT**

*Plasmodium* parasites are the causative agent of human malaria, a disease that despite global efforts of eradication still infects over 200 million people and kills hundreds of thousands of people every year. Transmission of this disease relies on the infectious bite of more than 50 different mosquito species of the genus *Anopheles* that occupy diverse geographic and ecological niches. This global distribution enables malaria transmission to occur on nearly every continent, yet not all species are alike in their capacity to efficiently transmit this parasite. Indeed, vectorial capacity shows remarkable interspecies heterogeneity due to a host of biological processes within the mosquito that can impact *Plasmodium* survival but also modulate vector fitness. Moreover, individual *Anopheles* lineages have experienced different degrees of pressure from human *Plasmodia* and their ancestral species due to geographic variation in disease burden over millions of years. Such heterogeneity may be reflected by different evolutionary signatures present in the mosquito genome, enabling the discovery of mosquito factors or molecular processes that may have been shaped by *Plasmodium* infection.

In this dissertation, we examine the response of *Anopheles gambiae*, the major malaria vector in sub-Saharan Africa, to infection with the deadliest human malaria parasite, *Plasmodium falciparum*. Given *An. gambiae*’s high reproductive rate and unique mating biology, we focus particularly on the reproductive fitness of this species in an effort to elucidate components of its biology that contribute to vectorial capacity. Through this work, we gain significant insight into molecular and physiological processes that impact *An. gambiae* fitness in the face of infection and may have been shaped by *P. falciparum*. Furthermore, we identify ecological factors that
affect the *An. gambiae*-*P. falciparum* interaction, potentially modulating mosquito resistance to infection and impacting transmission dynamics in the field.

After a review of the literature on the *Anopheles*-*Plasmodium* association in Chapter 1, in Chapter 2 we perform studies on the role of Mating-Induced Stimulator of Oogenesis (MISO), a female protein produced in reproductive tissues in response to sexual transfer of the steroid hormone 20-hydroxyecdysone (20E), in limiting the fitness costs inflicted by *P. falciparum* infections on female reproductive output. We first examine the reproductive fitness of *Anopheles* species from different geographical regions and from different evolutionary lineages infected with these malaria parasites. These analyses show that while important vectors like *An. gambiae* and *Anopheles stephensi* that have experienced considerable long-term pressure from *P. falciparum* do not suffer a cost to infection, the Central American vector *Anopheles albimanus* which has had a more recent association with this malaria species produces fewer eggs when fed on an infectious blood meal. Moreover, we determine that the tolerance to *P. falciparum* infection observed in *An. gambiae* is at least partially due to the function of MISO, and that MISO likely acts within 20E-mediated signaling cascades at the interface of blood feeding- and mating-induced pathways that maintain female reproductive fitness. As the evolution of MISO within the *Anopheles* genus appears to have been driven by differential transfer of male 20E during mating, specific evolutionary trajectories of post-mating processes in various *Anopheles* subgenera may have contributed to their differential ability to support *Plasmodium* parasites. Importantly, these studies reveal a previously unknown link between two 20E-regulated processes, hypoxia-induced molting and egg development, that may impact both reproductive success and *Plasmodium* development.

In Chapter 3, we turn our focus from tolerance to resistance mechanisms in *An. gambiae* by determining the effects of the endosymbiotic bacteria *Wolbachia* on *P. falciparum* and on its mosquito host. We find that the *Wolbachia* species *wAnga* that infects *Anopheles* populations from West Africa negatively correlates with *Plasmodium* in field-captured mosquitoes. Laboratory-
based studies, however, do not show a striking effect of wAnga on \textit{P. falciparum}, suggesting the ecological context of natural \textit{Anopheles} populations may be critical to wAnga’s potential anti-plasmodial effects.

This body of work expands our understanding of the molecular and physiological processes in \textit{Anopheles} that contribute to vectorial capacity and tolerance to infection, and highlights how ecological pressures imposed on natural populations affect parasite survival within the mosquito and may have shaped the evolution of vector-parasite interactions.
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CHAPTER 1:

Introduction

This chapter was written by Perrine Marcenac, with editorial input from Flaminia Catteruccia. Adam South performed the meta-analysis.
1.1 *Plasmodium* parasites as drivers of selection

Parasites of the genus *Plasmodium* are the causative agent of malaria, a disease with a global distribution that infected 216 million people and claimed the lives of 445,000 people in 2016 alone \(^1\) (Figure 1.1). The number of humans who have been infected with *Plasmodium* parasite since malaria first began infecting humans is on the order of billions, if not more, and in this time this parasitic infection has acted as one of the strongest selection pressures on human genetics. Indeed, malaria has been shown to have selected for erythrocyte variants at local and global scales, some of them within the last 10,000 years, that confer protection to malaria but that can also be deleterious in homozygous individuals (reviewed in \(^2\)). An intriguing but understudied question is whether *Plasmodium* has exerted similar selection pressure on the genome of its vector, mosquitoes of the genus *Anopheles*, as the *Anopheles-Plasmodium* association is far older than the human-*Plasmodium* interaction.

![Figure 1.1 | Malaria cases are distributed across the globe, with the greatest burden of infection in sub-Saharan Africa. Colors represent the confirmed cases of malaria per 1000 people at risk in 2015 \(^3\). The figure is adapted from \(^4\).](image-url)
1.2 A very long engagement: The interaction between *Plasmodium* parasites and *Anopheles* mosquitoes

Human malaria parasites are transmitted by the infectious bite of *Anopheles* mosquitoes, of which an estimated 70 species are competent vectors and occupy diverse geographic and ecological niches (Figure 1.2). The *Anopheles* genus is very old and broke off from the *Culicinae* lineage (containing *Aedes* and *Culex* mosquito species) about 217 million years ago (95% confidence interval: ~230-192 million years ago) (Figure 1.2). The last common ancestor of the three subgenera of *Anopheles* – *Cellia*, *Nyssorhynchus*, and *Anopheles* – was approximately 100 million years ago, “close” in time on an evolutionary scale to when the South American and African continents split apart over 115 million years ago. The geographic distribution of these three subgenera resulted in large part from this continental shift, with *Cellia* located in the Old World, *Nyssorhynchus* in Central and South America, and the *Anopheles* genus distributed globally from North America to Asia.

Figure 1.2 | *Anopheles* vectors competent to transmit *Plasmodium* malaria parasites are distributed globally and occupy diverse ecological niches. Figure adapted from 6.
How long have anophelines been transmitting *Plasmodium* parasites? The date of emergence of mammalian *Plasmodium* parasites remains controversial, with some estimates placing this event at around 13 million years ago \(^{10}\) and others at over 60 million years ago \(^{11}\). Regardless of the exact date of emergence of mammalian malaria, it is clear the *Anopheles* genus precedes the existence of mammalian *Plasmodium* parasites, and that anophelines have likely been transmitting these parasites for millions of years. But while the *Anopheles*-*Plasmodium* interaction is very old, anophelines began transmitting *Plasmodium falciparum*, the deadliest malaria parasite, quite recently. Analysis based on single-genome amplification of *Plasmodium* species infecting apes in central Africa revealed human *P. falciparum* originated in gorillas from *Plasmodium praefalciparum* parasites \(^{12}\). Estimates based on the nucleotide diversity, mutation rate, and number of parasitic replication cycles per year of *P. falciparum* place transmission to humans within the last 10,000 years \(^{12,13}\), though other studies show evidence that some lineages of *P. falciparum* could be 50,000-100,000 years old \(^{14}\). Following transmission to humans, *P. falciparum* then began spreading out of Africa with human migration, adapting to its many anopheline vector species along the way (reviewed in \(^{15}\)). While Old World vectors have been transmitting *P. falciparum* for thousands of years, New World anophelines have only recently encountered this parasite. *P. falciparum* was brought to the New World with the transatlantic slave trade starting in the 16\(^{th}\) century \(^{16}\); therefore, *Anopheles* vectors of the *Nyssorhynchus* subgenus have experienced evolutionary pressure from *P. falciparum* infection on a much shorter scale than Old World vectors.

### 1.3 Searching for signatures of *Plasmodium* selection in *Anopheles* genomes

There are numerous convincing lines of evidence suggesting *Plasmodium* has acted as a driver of selection in *Anopheles* genomes. First, the burden of *Plasmodium* infections in *Anopheles* mosquitoes has been significant on an evolutionary scale. To approximate this scale without a reliable anopheline fossil record, we can use present-day rates of infection in sub-
Saharan Africa where the burden of *Plasmodium* species has been the most significant since the dawn of the *Anopheles-Plasmodium* interaction. In a very recent survey of a malaria-endemic area in Burkina Faso, 49.77% of children 15 years old and younger tested for *Plasmodium* had positive blood smears \(^{17}\). In another study of a malaria-endemic village in Senegal, the authors reported that 98.6% of residents surveyed (including children and adults) were infected with *P. falciparum* at least once during the 4-month study \(^{18}\). With regards to mosquitoes, a 2008 survey examining the rate of infection with the *Plasmodium* transmission stages (sporozoites) in *Anopheles arabiensis* and *Anopheles gambiae sensu lato* (s.l.) — comprised of the species *Anopheles gambiae sensu stricto* (s.s.) and *Anopheles coluzzii* — in the city of Bobo-Dioulasso, Burkina Faso found infection rates below 6.2% across all sampled populations \(^{19}\). However, sporozoite rates underestimate the actual *P. falciparum* prevalence in anopheline populations, as a significantly greater proportion of mosquitoes must harbor the parasite for the majority of their lifetime in order for a subset of the population to survive to transmit infectious sporozoites. Transmission from mosquito to human must have been quite high for these malaria prevalence rates to be detected in humans, suggesting high malaria prevalence in the *Anopheles* vector as well.

The short generation time of *Anopheles* mosquitoes provide further support for *Plasmodium* shaping mosquito genetics. The human generation time is estimated around 25 years \(^{20}\), and even with this relatively lengthy generation time, *Plasmodium* infection has selected for erythrocyte variants within the last 10,000 years \(^{21-23}\). But within one human generation, *Anopheles* mosquitoes can have over 300 (with average anopheline generations of 1 month), scaling up the potential impact *Plasmodium* infection in mosquitoes could have on mosquito genomes.

Recent efforts to sequence the genomes of 16 *Anopheles* species sampled from wide geographical and ecological ranges and with different degrees of vectorial capacity have revealed variation across these genomes in a number of processes related to vector competence, including
immunity genes and reproductive traits. With these studies, we can begin to elucidate factors that might have experienced selective pressure from *Plasmodium* by 1) examining processes within the mosquito that interface with *Plasmodium* development, and 2) studying traits important for vector competence that differ between species that have experienced high versus low levels of pressure from *P. falciparum* infection.

1.4 *Plasmodium* infection in the *Anopheles* mosquito

*An. gambiae* s.l. is the primary vector species of *P. falciparum* in sub-Saharan Africa, where *P. falciparum* accounts for 99% of all malaria cases in that region. Like all anautogenous mosquito species, *An. gambiae* females require a blood meal for egg development, or oogenesis, to occur, but in the course of this bloodmeal, female mosquitoes may also become infected with *P. falciparum* if they feed on an infected human host. The first 48 hours following a bloodmeal are absolutely critical for both the parasite and the mosquito (Figure 1.3). During this time, *P. falciparum* must successfully exit the midgut epithelium and establish itself underneath the basal lamina while avoiding the mosquito’s production of immune modulators (Figure 1.3a), while the female mosquito must successfully digest the bloodmeal and produce eggs to ensure its reproductive fitness (Figure 1.3b).

When *An. gambiae* females take a *P. falciparum*-infected blood meal, the sexual stages of the parasites, called gametocytes, enter the mosquito midgut, where a drop in temperature and the presence of xanthurenic acid stimulate gametogenesis, yielding male and female gametes. These gametes can then fuse to form a diploid zygote which differentiates into an ookinete that utilizes gliding motility to move within and out of the blood meal, which by the time of ookinete formation is contained by a peritrophic matrix secreted by the midgut epithelium. Within 48 hours following a blood meal, ookinetes cross the midgut epithelial cells, entering at the apical membrane and egressing from the basal side, where they become sessile upon contact with the basal lamina and round up into oocysts (reviewed in). Here, oocysts grow over the next
8-12 days and undergo sporogony, a large-scale asexual amplification of the parasite genome, forming sporozoites. Approximately 12-14 days following the infectious bloodmeal, the oocysts begin to rupture, releasing thousands of sporozoites into the mosquito hemolymph (circulatory system) that can then invade the mosquito salivary glands by gliding motility, where they can then penetrate into the secretory ducts to be transmitted to a human host at the next blood meal a female mosquito takes (Figure 1.3a).

During the first 48 hours after P. falciparum gametocytes enter the mosquito midgut, Anopheles mosquitoes produce anti-plasmodial factors capable of detecting and/or killing parasites. These factors have been studied at length and vary depending on whether mosquitoes (usually An. gambiae or Anopheles stephensi) are infected with the human P. falciparum parasite or the mouse model parasite Plasmodium berghei. An. gambiae females mount a potent immune response to P. berghei infection mediated by the complement-like factor Thioester-containing protein 1 (TEP1) which when stabilized by two proteins of the leucine-rich repeat family, LRIM1 and APL1C, effectively lyases ookinetes that have crossed the midgut epithelium and come in contact with mosquito hemolymph. It is hypothesized that this TEP1-mediated lysis occurs in part because nitration in infected midgut cells mediated by nitric oxide synthase and heme peroxidase enzyme activity “marks” parasites for lysis. Expression of TEP1 and these nitration factors is under the control of the JNK pathway, while TEP1 and LRIM1 are further regulated by the NF-κB transcription factors REL1 and REL2. Although these same pathways are also important in mediating the immune response to P. falciparum infection, there is remarkable variation in the effect of silencing of these immune effectors on P. falciparum infection in different Anopheles species depending on the mosquito species and parasite strain used. For example, silencing of TEP1 in An. gambiae females infected with single clones of P. falciparum parasite from field-collected isolates increased oocyst intensities in these females, but this effect was abolished when females were infected with multiclonal field isolates. In another study, knock-down of TEP1 expression in An. gambiae mosquitoes did not impact infection with the
NF54 strain, an African isolate of *P. falciparum*; however, when these TEP1-silenced females were fed blood infected with the Brazilian *P. falciparum* strain 7G8, they were no longer capable of mounting a potent immune response to the parasite. This diversity is hypothesized to be in part due to *P. falciparum* having evolved a mechanism mediated by the surface protein Pfs47 to evade nitration and the complement-like system. Pfs47 haplotypes were found to have significant geographical differentiation, and the haplotypes from a given geographical region were shown to contribute to *P. falciparum* immune-evasion in the *Anopheles* vector from that same region, but not in *Anopheles* species from a different geographical region. These data suggest that *P. falciparum* has adapted to its vector in order to ensure maximal parasitic survival and transmission.

Beyond nitration and the mosquito complement-like system, other factors can impact *Plasmodium* survival in the mosquito midgut. Reactive oxygen species (ROS) produced following blood feeding increase upon *P. berghei* infection and contribute to parasite killing, and while no similar upregulation following *P. falciparum* infection has been demonstrated, *P. falciparum* was shown to suppress the production of ROS as a mechanism of immune avoidance. Taken together, the production of these immune effectors within the first 48 hours following blood feeding can greatly impact *P. falciparum*’s invasion of the midgut epithelium.
Figure 1.3 | \textit{P. falciparum} development and oogenesis are physiologically and temporally tied in \textit{Anopheles} mosquitoes. Within 48 hours of taking a bloodmeal, \textit{An. gambiae} females will develop eggs that they can lay provided they are mated. If the bloodmeal contains the sexual stages of \textit{P. falciparum} parasite, then over the next 48 hours as egg development occurs in \textit{An. gambiae}, the parasite will undergo major developmental steps leading to oocyst establishment in the midgut epithelium.

1.5 Hormonal regulation of \textit{Anopheles} oogenesis following blood feeding

After blood feeding, female \textit{An. gambiae} develop eggs within 48 hours (Figure 1.3b), but the signaling pathways essential for this process are still poorly understood in \textit{Anopheles} species. They have been more comprehensively characterized in the dengue virus mosquito vector, \textit{Aedes aegypti}. In this species, blood feeding initiates the release of ovarian ecdysteroidogenic hormone
(OEH) and insulin-like peptide (ILP) family members, including ILP3, from medial neurosecretory cells in the brain, which in turn stimulates the production of ecdysone hormone (E) from the ovaries (Figure 1.4). E is then hydroxylated to 20-hydroxyecdyadysone (20E) in the fat body, the liver-like organ of mosquitoes, where it then stimulates transcription of yolk protein precursors (YPPs), including vitellogenin (Vg) and lipophorin (Lp), in the fat body by binding to the nuclear hormone receptor heterodimer ecdysone receptor (EcR)/ultraspiracle (USP), inducing its function as a transcriptional activator. These YPPs then travel in the hemolymph and are taken up by the ovaries via receptor-mediated endocytosis. The activity of OEH, ILPs, and E has been shown to be enhanced by amino acid sensing through the target of rapamycin (TOR) pathway (Figure 1.4). 20E has a similar effect on vitellogenesis (yolk formation) following a blood meal in An. gambiae, and several molecular factors important for oogenesis are present in the Anopheles genome, suggesting that these pathways are conserved. However, no published studies have directly examined the role of OEH, ILPs, and TOR signaling on egg development in An. gambiae.
Figure 1.4 | Signaling and hormonal pathways activated when female mosquitoes consume blood. In *Ae. aegypti*, bloodmeal stimulates females to produce OEH and ILPs in the brain, which then signal to the ovaries to produce ecdysone hormone. Ecdysone is hydroxylated to 20E in the fat body, in turn stimulating the expression of the YPPs *Vg* and *Lp* which go on to transport lipids to the developing ovaries. A nutritional signal mediated by TOR enhances this hormonal signaling. These signaling pathways are likely conserved in *Anopheles* mosquitoes.

1.6 20E as the master regulator of *An. gambiae* mating biology

While *An. gambiae* females require a blood meal to develop eggs, they must be mated to oviposit these eggs and generate fertile progeny. This mating event occurs when females are attracted to swarms formed at dusk by male mosquitoes. Upon copulation, males transfer seminal secretions including sperm, stored in the female’s sperm-storage organ or spermatheca, and a coagulated mating plug that enters the female atrium, or uterus, where it is broken down over the next 24 hours. This mating plug is a structure rich in lipids, proteins, and strikingly, the steroid hormone 20E. Sexually-transferred 20E then acts as a signal to induce a host of
post-mating phenotypes including refractoriness to further mating, oviposition behavior\textsuperscript{68}, proper sperm storage\textsuperscript{69}, and an increase in female fecundity (number of eggs developed)\textsuperscript{70}.

The 20E transferred during mating impacts blood feeding-induced processes (Figure 1.5). Mated \textit{An. gambiae} females produce higher titers of 20E following blood feeding compared to virgin controls (E. G. Kakani and S. N. Mitchell, unpublished). The mechanism underlying this increased 20E production is still unknown but could be due to 20E released from the mating plug priming the fat body and/or ovaries to be more competent to produce ecdysteroids following blood feeding. In support of this hypothesis, mated females produce more transcripts of the YPP \textit{Lp} in their fat bodies following blood feeding relative to virgin blood-fed females\textsuperscript{70}. This increased \textit{Lp} transcription is mediated by the female protein Mating-Induced Stimulator of Oogenesis (MISO) that is produced following mating in the atrium in a 20E-dependent manner, interacts in a complex with 20E, and transduces this signal into increased egg development in \textit{An. gambiae} \textsuperscript{70}. Furthermore, females depleted of MISO via RNA interference (RNAi) show impaired lipid accumulation in their ovaries\textsuperscript{70}. These data demonstrate that mating-transferred 20E impact and potentiate blood meal-induced signaling (Figure 1.5).
**Figure 1.5 | 20E transferred during mating impacts blood feeding-induced processes.** Through still unknown mechanisms, mating increases the amount of 20E produced post-blood feeding. Mated blood-fed females produce higher levels of the YPP Lp, and this increase is mediated by the female protein MISO that is produced in a 20E-dependent manner in the atrium post-mating.

While the pathways underlying oogenesis following blood feeding are likely conserved among anophelines (given evidence of conservation of these factors between *Aedes* and *Anopheles*), mating biology and mating-induced processes have greater heterogeneity within this genus. Although many anophelines mate in swarms and exhibit female monandry (females mate with only one male in their lifetime), not all species in this genus receive a coagulated mating plug during mating. In fact, the transfer of a mating plug and the male-synthesized steroid hormone 20E is a derived trait that has evolved specifically in some anopheline species from an ancestor that did not transfer 20E or a plug (*Figure 1.6*) \(^{71}\). The African vectors of malaria, including *An. gambiae*, *An. arabiensis*, and *Anopheles funestus*, as well as the Indian species *An. stephensi*, all transfer a fully coagulated mating plug containing high levels of 20E, while Asian (*Anopheles dirus*, *Anopheles farauti*, and *Anopheles sinensis*) and European (*Anopheles*
atroparvus) species have a partially coagulated plug and low to intermediate levels of 20E \(^7\). Strikingly, males of the Central American vector *Anopheles albimanus* whose ancestor branched off from the *Cellia* and *Anopheles* subgenera 100 million years ago \(^8\) do not transfer a plug or 20E to females during mating (**Figure 1.6**) \(^7\). Evidence of reciprocal adaptation in females of species that receive 20E in the plug was demonstrated by performing evolutionary analysis on the female protein MISO. While MISO is highly divergent among the anophelines, sequence similarity between species grouped more according to the relative titer of 20E transferred during mating rather than according to the *Anopheles* phylogeny \(^7\).

Only a subset of anophelines acquired the trait of plug and 20E transfer during mating, and this transfer drove the reciprocal adaptation of MISO \(^7\), a protein which has been demonstrated to affect blood feeding processes and confer a fitness-boosting phenotype \(^70\). The species that have evolved this unique mating biology are restricted primarily to the African continent, with more diluted levels of these phenotypes (in transferred 20E and plug coagulation levels) as the species move further away from Africa. This geographic distribution of plug and sexually-transferred 20E phenotypes is remarkably reminiscent of the distribution of *P. falciparum* transmission (see **Figure 1.1** and **Figure 1.6**), suggesting the intriguing hypothesis that evolution of this fitness trait may have impacted or been affected by *P. falciparum*. This is a particularly compelling hypothesis considering *P. falciparum* has been shown to utilize blood feeding-induced signaling cascades for its own development.
Figure 1.6 | Ancestral state reconstructions of mating plug and 20E characters. Using the mating plug phenotypes (left) and the levels of 20E (right) of 9 Anopheles species and 2 related species of the order Diptera (Ae. aegypti and Drosophila melanogaster), maximum parsimony ancestral state reconstructions were performed. This analysis revealed that the most common ancestor lacked a plug and 20E in the male accessory glands (MAGs), indicated with stars. The capacity to form a plug arose after the An. albimanus lineage split off from the rest of the Anopheles (node A), and the production of 20E in the MAGs occurred after node C. The colors of the species names correspond to geographical location. Figure adapted from 71 and reprinted with permission from AAAS.
1.7 An expert hijacker: how *Plasmodium* utilizes mosquito blood feeding-induced factors for its own development

In light of its reliance on multiple blood feeding events for its transmission, it is evident that *Plasmodium* would benefit from utilizing nutrients that are naturally produced to promote oogenesis. Indeed, evidence points to *Plasmodium* utilizing Vg and Lp, nutrient-carrying proteins that transport lipids to the developing ovaries, for its own development. In *P. berghei* infections, Vg and Lp were shown to interfere with TEP1 binding to the ookinete. The mechanism by which these YPPs affect TEP1, however, is unknown. Lp was recently shown to directly provision developing *P. berghei* oocysts with lipids, and silencing of this gene reduced oocyst size, sporulation, and infectivity of sporozoites. Lp knock-down in *An. gambiae* infected with *P. falciparum* also reduced mean oocyst intensities, mean oocyst size, and the infectivity of sporozoites relative to controls, though whether this effect is mediated by reduced lipid trafficking to *P. falciparum* oocysts has yet to be conclusively demonstrated. *P. falciparum* infection was also shown to increase the production of the insulin-like peptides ILP2, ILP3, ILP4, and ILP5 post-blood feeding in *An. stephensi*. Repression of ILP3 and ILP4 increased the expression of nitric oxide synthase, APL1, TEP1, and LRIM1 and decreased *P. falciparum* prevalence and intensity. Whether these phenotypes are the result of a direct manipulation operated by the parasite on mosquito metabolic factors or rather an indirect effect from the mosquito’s response to infection is unknown. Taken together, these data show *P. falciparum* can utilize blood feeding-induced factors for its own development, but none of these studies demonstrated whether this interaction is truly parasitic and induces a fitness cost in mosquitoes.

1.8 Tolerance and resistance: How do anophelines survive with *Plasmodium* infection?

As mentioned above, *Anopheles* female reproductive fitness relies on a complex biology that involves blood feeding-induced but also mating-induced signaling. Given that females may become infected with *Plasmodium* parasites in the course of essential reproductive processes, it
is reasonable to speculate that Anopheles vectors would benefit from having evolved mechanisms to control infection by limiting Plasmodium virulence or reducing costs associated with parasite development. These strategies in response to a parasitic infection are known as resistance and tolerance. Resistance involves mechanisms to control and/or reduce infection, while tolerance does not limit infection but rather reduces fitness costs associated with it. Mechanisms underlying resistance are relatively well-characterized and usually involve the immune system detecting and then killing pathogens; however, the mechanisms underlying tolerance are far less understood but provide a fascinating look into the evolution of host-pathogen interactions. These mechanisms can take many forms and can involve intrinsic genetic factors in the host, or extrinsic factors from the host’s environment. For example, zebra fish produce an intestinal alkaline phosphatase that detoxifies LPS in the gut in order to avoid mounting a costly immune response to their own microbiome, conferring tolerance. Another study demonstrated that Aedes albopictus mosquitoes infected with Chikungunya virus produce viral-derived DNA (vDNA) generated from endogenous reverse transcriptase activity that promotes host survival in the face of infection, as treatment of mosquitoes with a blocker of endogenous reverse transcriptase activity to reduce vDNA levels did not affect Chikungunya viral load but reduced mosquito survival. Organisms can also utilize ecological factors to promote tolerance, as was demonstrated in the monarch butterfly Danaus plexippus that can reduce the life-shortening effects of infection with its naturally occurring protozoan parasite Ophryocystis elektroscirrha by consuming the milkweed species Asclepias curassavica, rather than other milkweed species.

Anopheles mosquitoes possess resistance mechanisms to parasitic infection as evident from a number of studies that have elucidated the potent immune factors that can kill Plasmodium, including the mosquito complement-like system, production of ROS, the gut nitration response, and stimulation of NF-κB transcription factors. However, P. falciparum has in turn adapted to its anopheline vectors and has gained the capacity to evade these resistance mechanisms via strategies including production of the surface protein Pfs47. Regardless of this
capacity to evade the immune system, *P. falciparum* still requires that infected females survive a minimum of 12-14 days after an infected bite to ensure its transmission. Furthermore, over generations and generations of transmission, the parasite would also benefit from not inducing reproductive trade-offs in the vector that could potentially reduce the size of the *Plasmodium*-susceptible population. These observations lead to the testable hypothesis that *P. falciparum* does not induce fitness costs in its natural vector species.

To determine if anophelines suffer fitness costs or have evolved tolerance mechanisms to *Plasmodium* infection, the longevity and reproductive output of *Plasmodium*-infected mosquitoes relative to uninfected controls must be assessed. A 2002 meta-analysis on 11 published studies examining the effect of parasite development on mosquito survival found that in *Plasmodium* infections of mosquitoes that are natural vectors of a particular *Plasmodium* species (referred to as natural *Anopheles-Plasmodium* associations from this point onward), no costs to survival associated with infection were detected. Specifically, when *An. gambiae*, *An. funestus*, and *An. stephensi* were infected with the human malaria *P. falciparum*, their lifespan was unaffected; however, *An. stephensi* showed reduced survival when infected with the rodent malarias *P. berghei*, *Plasmodium yoelii nigeriensis*, and *Plasmodium chabaudi*. Additionally, our own unpublished meta-analysis of 13 studies that examined potential trade-offs in the fecundity of *Anopheles* species infected with different species of *Plasmodium* gave similar results. In natural *Anopheles-Plasmodium* associations, no negative effects to female fecundity were recorded in all studies except for one (Figure 1.7, left section), while most non-natural vector-parasite combinations induced significant costs on mosquito fecundity, as shown by effect sizes (Figure 1.7, right section). Future studies will need to examine the effect of *Plasmodium* infection on female *Anopheles* fertility, another key component of reproductive fitness, as well as potential effects of infection across multiple blood feeding cycles. Taken together, these two meta-analyses provide strong evidence suggesting that *Anopheles* mosquitoes have evolved tolerance mechanisms to their *Plasmodium* parasites, though these mechanisms have yet to be elucidated.
Figure 1.7 | Effects of *Plasmodium* infection on *Anopheles* fecundity. In a meta-analysis of 13 published studies, infection with *Plasmodium* does not induce a negative effect on fecundity in natural *Anopheles*-*Plasmodium* associations (left section, effect size ≥ 0), whereas non-natural associations introduce a reproductive cost in the majority of recorded cases (right section, effect size < 0). For each comparison, we calculated standardized mean difference as Hedge’s d, an unbiased weighted estimate of effect size that is calculated as the difference between a control and experimental group measured in standard deviation units. In this meta-analysis, the control was always defined as the group in which females fed on uninfected blood, while the experimental group was defined as the group in which females fed on infected blood. MetaWin 2.1⁹⁴ was used for effect size calculations. The numbers above the bars represent reference numbers.

1.9 Ecological factors could shape *Anopheles* tolerance to *Plasmodium* infection

Genetic factors underlying mosquito reproduction and immunity, including Lp, Vg, TEP1, and other proteins involved in the complement-like system, directly impact *Anopheles* resistance
mechanisms to *Plasmodium* infection. Some of these genetic factors may also affect tolerance mechanisms, though this has yet to be thoroughly investigated. But besides their genetic components, tolerance and resistance mechanisms must also be considered in relation to the ecological and environmental context characterizing specific *Anopheles-Plasmodium* interactions. Indeed, a host of non-genetic factors have been shown to impact *Plasmodium* infection within the mosquito, including temperature, larval and adult diet, competition in larval breeding sites, and the anopheline microbiome (reviewed in [95](#)). These factors may play an important role in tolerance to infection, considering that mounting an immune response can be costly to the host [96](#) and that the level of this cost depends strongly on the environment [97](#). In support of this hypothesis is a study in *An. coluzzii* demonstrating that feeding on the nectars of plants present in their natural habitat can differently impact *P. falciparum* infection intensity and prevalence [98](#). Females fed on the local flowering plant species *Thevetia neriifolia* prior to and after infection had a lower sporozoite index (calculated as the proportion of females with sporozoites present in their salivary glands 14 days post-infection) relative to control females fed on 5% glucose, while females fed on another flowering plant *Barleria lupilina* showed the opposite effect [98](#). Further evidence that ecological factors can influence vector competence can be found in the multiple studies showing that *Plasmodium* infections are deeply regulated by the mosquito microbiome, as discussed in the next section.

**1.10 The *Anopheles* microbiome affects *P. falciparum* infection**

*Plasmodium* is not alone when it colonizes the *Anopheles* midgut: this digestive tissue harbors a rich microbiota that can impact parasite survival and development and thus, vector competence. Following blood feeding, the natural midgut microbiota of *An. gambiae* dramatically proliferates [99](#) and can control *P. falciparum* infection, as aseptic mosquitoes harbor increased oocyst intensities relative to septic controls [100](#). This is in part due to the bacteria stimulating the transcription of immune regulators, including peptidoglycan recognition proteins, pattern
recognition receptors, anti-microbial peptides, and serine proteases, which in turn can limit *P. falciparum* development\(^{100,101}\). Furthermore, specific bacterial species present in the gut microbiome can limit *P. falciparum* development when *An. gambiae* are infected with additional titers of these bacteria by stimulating the production of anti-parasitic factors, such as ROS\(^ {102}\).

While most of these studies have examined how the production of immune modulators by the anopheline microbiota impact *Plasmodium* survival, host-pathogen studies in other systems, including infection of *Aedes aegypti* with dengue, Chikungunya, and *Plasmodium*, suggest that resource competition between bacteria and pathogens may also mediate bacterial pathogen-killing effects\(^ {103,104}\).

Far less studied than the midgut, the reproductive tissues of anopheline vectors are also populated with a diverse microbiome\(^ {105}\) and host bacterial species that could be utilized for anti-*Plasmodium* activity\(^ {106-108}\). Due to their capacity to be vertically transmitted from mother to offspring and to propagate in tissues relevant to *Plasmodium* transmission (midgut and salivary glands) in addition to reproductive tissues, some of these endosymbionts, including *Asaia*\(^ {107,109}\) and *Serratia*\(^ {108}\), have been proposed as candidates for paratransgenesis – the use of genetically-modified bacteria carrying anti-plasmodial cargoes – to reduce vector competence. Indeed, a recent study modified naturally-occurring *Serratia marcescens* to secrete anti-*Plasmodium* effector proteins in *An. gambiae* mosquitoes, effectively reducing *P. falciparum* oocyst loads\(^ {108}\).

A prominent bacterial species that has attracted considerable attention as an anti-pathogenic endosymbiont is the vertically-transmitted alpha-proteobacterium *Wolbachia*. *Wolbachia* is an obligate intracellular bacterium that in some of its insect hosts, most notably *Aedes* mosquitoes, can rapidly invade the host population through a process known as cytoplasmic incompatibility (CI) (reviewed in\(^ {110}\)) (Figure 1.8). CI prevents uninfected females from producing viable progeny after mating with *Wolbachia*-infected males due to chromosomal segregation defects in the early cellular divisions of the fertilized egg. However, infected females are able to produce viable progeny following matings with uninfected and infected males which
then favors Wolbachia's spread \(^{111,112}\) (Figure 1.8). Recent studies have shown that proteins encoded by the Wolbachia prophage WO induce CI, though the mechanism by which they cause these chromosomal defects is still unknown \(^{113,114}\). In addition to possessing mechanisms to drive itself into a host population, Wolbachia has also been shown to increase insect host resistance to a number of vectorial pathogens including Plasmodium \(^{103,115-117}\). The mechanism by which Wolbachia induces pathogen resistance is still under active investigation, but studies suggest these endosymbionts can boost mosquito innate immunity, including NF-κB transcription factor signaling \(^{103,115,116,118,119}\), and can compete with the pathogen for resources \(^{103,104,120,121}\). Increased production of ROS and antimicrobial peptides have been documented in both naturally infected \(^{122}\) and transinfected \(^{117,123}\) mosquito species. However, increases in host immune responses in both Ae. aegypti \(^{118,119,124}\) and An. gambiae \(^{115,116}\) that lead to pathogen resistance may in fact result from recent infection of these new hosts with non-native Wolbachia \(^{123}\). The recent identification of natural Wolbachia infections in An. gambiae, An. coluzzii \(^{106}\), and An. arabiensis \(^{125}\) provides the unprecedented opportunity to investigate whether Wolbachia uses reproductive manipulation to drive itself into natural anopheline populations and whether it confers protection to P. falciparum infection, which would have tremendous implications for the Anopheles-Plasmodium interaction and maintenance of tolerance to infection (see Chapter 3).
Figure 1.8 | *Wolbachia* infection can rapidly invade the host population by inducing CI. Shown here in *Ae. aegypti*, crosses between *Wolbachia*-infected males and uninfected females produce egg batches with high infertility due to chromosomal segregation defects in the early cellular divisions of the fertilized eggs, yielding few viable progeny. However, infected females mated with uninfected or infected males produce normal, fertile progeny.

1.11 Concluding remarks

The co-evolution of *Anopheles* mosquitoes and *Plasmodium* parasites has been a dynamic process over millions of years, modulated by adaptation in both the vector and the parasite to limit deleterious effects associated with infection but also promote parasite survival, respectively. Given the strength of malaria as a selective force on human genetics on a significantly smaller timescale, it is inarguable that *Plasmodium* has also shaped the genome of its *Anopheles* vectors. Searching for anopheline factors or processes that may have been selected for or co-opted by *P. falciparum*, and understanding how ecological factors, including the mosquito microbiome, may have shaped the *Anopheles*-*P. falciparum* interaction will provide valuable insights into *Anopheles* vectorial capacity.
1.12 Summary of aims

The work described in this dissertation examines the evolution of vector-parasite interactions with a focus on anopheline reproductive fitness. While a number of An. gambiae resistance mechanisms to P. falciparum infection have been elucidated, we seek to address the gap in knowledge on tolerance mechanisms that may have evolved in response to Plasmodium parasites over millions of years of co-evolution. We also seek to elucidate ecological factors that may have promoted An. gambiae resistance to P. falciparum infection, potentially enabling greater investment in tolerance mechanisms.

In Chapter 2, we examine whether infection with P. falciparum induces costs to reproductive fitness in three vector species selected from different geographic areas: An. gambiae, An. stephensi, and An. albimanus. We demonstrate that the African species An. gambiae and the Indian vector An. stephensi of the Cellia subgenera incur no costs to their fecundity associated with infection, while the Central American species An. albimanus of the Nyssorhynchus subgenera experiences a significant reduction in fecundity associated with infection. We also show that in the natural An. gambiae-P. falciparum association, the number of oocysts produced following infection is positively correlated to the number of eggs developed per female. We provide evidence that one factor mediating this tolerance to P. falciparum infection in An. gambiae is the production of the 20E-induced protein MISO, a fitness-conferring factor that has reciprocally adapted to the transfer of 20E hormone in the mating plug in a subset of anophelines. We demonstrate that MISO, in addition to being expressed following mating in the atrium, is also produced in the ovaries and the trachea (respiratory organs) of females following blood feeding in a 20E-dependent way, and may be involved in development-promoting signaling mediated by hypoxia-induced signaling cascades post-mating and blood feeding. We hypothesize that millions of years of pressure from Plasmodium species in Old World anophelines may have contributed to the evolution of a 20E-based mating system which acts as a tolerance mechanism to infection in these species.
In Chapter 3, we investigate the impact of ecological factors on vector-parasite interactions by studying the effects of the alpha-proteobacterium *Wolbachia* on *Anopheles* reproduction and *Plasmodium* infection. We show that an *Anopheles*-specific *Wolbachia* species (called wAnga) that stably infects *An. coluzzii*, *An. gambiae* s.s., and *An. arabiensis* in natural populations in Burkina Faso does not induce CI in *An. coluzzii*, but does stimulate females to lay their eggs earlier. Furthermore, the presence of wAnga infection in *An. coluzzii* negatively correlates with *Plasmodium* infection in field-captured mosquitoes, suggesting *Wolbachia* can limit *Plasmodium* survival and growth in anophelines. Laboratory-based infections did not confirm this negative effect, suggesting that additional ecological factors absent in insectary conditions may influence the *Wolbachia*-*Plasmodium*-*Anopheles* interaction. Finally, we develop a model demonstrating that *Wolbachia* infection may be actively impacting malaria transmission dynamics in sub-Saharan Africa.

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1.14 References


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CHAPTER 2:
The female reproductive protein MISO regulates tolerance to *Plasmodium falciparum*
infection in *Anopheles gambiae*

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**Author Contributions:** \textbf{P.M.} and \textbf{F.C.} designed the study. \textbf{P.M.} performed all the experiments and wrote the manuscript, with editorial input from \textbf{F.C.} \textbf{P.M.} and \textbf{F.C.} analyzed the data. \textbf{A.S.} performed the statistical analyses generating the Generalized Linear Model and logistic regressions of field-collected data. \textbf{V.A.I.} and \textbf{H.R.} designed the R Studio application to generate correlation networks and performed the correlation network analysis. \textbf{P.M.}, \textbf{E.G.K.}, and \textbf{S.N.M} determined the fecundity of *An. gambiae*, *An. stephensi*, and *An. albimanus* in the laboratory. \textbf{P.M.} and \textbf{D.G.A.} did the confocal microscopy experiments. \textbf{T.F.} and \textbf{S.R.Y.} conducted the experimental infections of *An. gambiae* with blood from gametocyte donors in collaboration with \textbf{A.D.}, and \textbf{W.R.S.} assisted in collecting egg and oocyst data from these field-generated studies.
ABSTRACT

The deadly malaria parasite *Plasmodium falciparum* depends on *Anopheles* mosquitoes for transmission to humans, and as such, the parasite would benefit from selecting for traits in its vectors that increase the likelihood of transmission. In particular, *P. falciparum* could exert selection pressure on molecular processes related to mosquito fitness in order to minimize potential negative effects associated with infection which could over time reduce the parasite-susceptible population. Here we investigated whether *Anopheles* species from different geographical locations and exposed to different degrees of evolutionary pressure from *Plasmodium* parasites suffer reproductive costs to infection. Egg development after blood feeding was not affected by *P. falciparum* infection in the African species *Anopheles gambiae* and Indian species *Anopheles stephensi*, while it was impaired in the Central American species *Anopheles albimanus*. Further studies in *An. gambiae* showed that sexual transfer of the steroid hormone 20-hydroxyecdysone (20E) affects female tolerance to *Plasmodium* infections, as silencing of the mating-induced and 20E-sensitive gene *Mating-Induced Stimulator of Oogenesis (MISO)* leads to decreased egg production in *P. falciparum*-infected females relative to uninfected and infected controls. We find that MISO is also produced in the ovaries of blood-fed females in a 20E-dependent manner, and that it localizes both to the ovarian oocyte and to trachea oxygenating the ovaries. Correlation network and transcriptional analysis suggest MISO is involved in conserved molting and oxygen-sensing signaling pathways previously not known to be involved in egg development in *An. gambiae*. Evidence that the 20E-MISO interaction mediates tolerance to *P. falciparum* infection in this major malaria vector suggests the parasite may have selected for or benefited from the evolution of sexual transfer of 20E to ensure its transmission.
INTRODUCTION

Mosquitoes of the genus *Anopheles* are the only vectors capable of transmitting *Plasmodium* malaria, a disease that infects hundreds of millions of people and was responsible for the deaths of over 400,000 people in 2016. These mosquitoes are distributed across the globe and fall into three major subgenera whose last common ancestor dates to 100 million years ago: *Cellia* found in the Old World, *Nyssorhynchus* located in Central and South America, and *Anopheles* distributed globally from North America to Asia. The many species within these three subgenera diverge in their efficiency to function as vectors for *Plasmodium falciparum*, the deadliest malaria species, based on a number of factors inherent to their biology that are relevant for pathogen transmission including longevity, reproductive fitness, and immune response mounted against the parasite.

Among the many anopheline species that transmit malaria, *Anopheles gambiae* are the main vectors of *P. falciparum* in sub-Saharan Africa, in part due to their significant reproductive rates that ensure high mosquito densities for parasite transmission. Egg development in these mosquitoes relies on two stimuli – blood feeding and mating – and both components are regulated by the molting steroid hormone 20-hydroxyecdysone (20E). Blood feeding induces 20E production in the female fat body (the liver-like mosquito organ), which in turn stimulates the transcription of the yolk protein precursors (YPPs) vitellogenin (*Vg*) and lipophorin (*Lp*). These YPPs then provision developing eggs with lipids (vitellogenesis) which will develop over 48 hours and will be laid provided the female is mated. During mating, *An. gambiae* males transfer seminal secretions including a coagulated mating plug rich in lipids, proteins, and the steroid hormone 20E. This sexually-transferred 20E induces a large-scale transcriptional response in the female reproductive organs that is responsible for a post-mating “switch,” whereby females are no longer susceptible to mating and are triggered to oviposit their eggs post-blood feeding. Furthermore, 20E transferred during mating impacts blood feeding processes, as mated females produce more eggs post-blood feeding relative to virgins via the function of the female protein...
Mating-Induced Stimulator of Oogenesis (MISO) \(^5\). We have previously reported that the expression of MISO is strongly induced in the female atrium by transfer of 20E 24 hours post-mating (hpm), and that MISO interacts with 20E to increase lipid transporter expression, enabling proper accumulation of lipids in the eggs and increasing the fecundity of females relative to virgin blood-fed females \(^5\).

While 20E-mediated signaling post-blood feeding is likely conserved across all anophelines given genetic conservation of blood meal-induced factors between *Anopheles* and another blood feeding genus *Aedes* \(^14\), within *Anopheles* the sexual transfer of a mating plug and male-produced 20E is a derived trait that evolved in only a subset of species \(^15\). Indeed, females of the African vectors of malaria, including *An. gambiae*, *Anopheles arabiensis*, and *Anopheles funestus*, as well as the Indian species *Anopheles stephensi*, all receive a fully coagulated mating plug containing high levels of 20E \(^15\). Asian (*Anopheles dirus*, *Anopheles farauti*, and *Anopheles sinensis*) and European (*Anopheles atroparvus*) species have less pronounced levels of this phenotype, with a partially coagulated plug and low to intermediate levels of 20E \(^15\). Strikingly, the Central American vector *Anopheles albimanus* whose ancestor branched off from the *Cellia* and *Anopheles* subgenera (containing the African, Asian, and European vectors) 100 million years ago \(^2\) does not transfer coagulated secretions or 20E during mating \(^15\). Evolutionary analysis on the MISO protein sequence suggested that male-transferred 20E has driven the reciprocal adaptation of MISO. While MISO is highly divergent within the *Anopheles* genus, sequence similarity between species clusters according to the amount of 20E transferred in the plug, rather than along the species phylogeny \(^15\). These data provide evidence that the evolution of this reproductive trait in males has shaped the evolutionary trajectory of female reproductive factors and, as a consequence, has influenced blood feeding-regulated processes. Other yet unknown co-adaptations are likely to have been triggered by the evolution of sexual transfer of 20E given this steroid hormone is a potent transcriptional activator inducing and repressing hundreds of genes after mating and blood feeding. With time, divergent evolutionary trajectories of female
post-mating and post-blood feeding physiology across anopheline species may have differentially affected their ability to support *Plasmodium* transmission.

*An. gambiae* females that ingest *P. falciparum* parasites when taking a blood meal for egg development can activate immune responses that reduce infection. The bulk of these responses occur in the mosquito midgut, where *P. falciparum* male and female gametes fuse to form a zygote that differentiates into a motile ookinete. This ookinete must successfully evade immune factors in the gut, including nitration\(^\text{16,17}\), reactive oxygen species (ROS)\(^\text{18,19}\), and the mosquito complement-like system\(^\text{20-23}\) in order to traverse the midgut epithelium and round up into oocysts upon contact with the basal lamina. Several studies have demonstrated that *Plasmodium* has exploited blood feeding processes to ensure its survival in the mosquito, as the same YPPs essential for anopheline egg development can be coopted by the parasite\(^\text{24,25}\) to reduce the efficiency of killing mediated by the mosquito complement-like system\(^\text{25}\) or to provision developing oocysts with lipids\(^\text{26,27}\).

Given that *Anopheles* mosquitoes can mount an immune response to *P. falciparum* infection and that the parasite can utilize mosquito factors for its own development, resource allocation models predict that *Anopheles* have less resources to devote to reproduction and general somatic processes if they are infected with *P. falciparum* (reviewed in\(^\text{28}\)). As a result, parasite-infected *Anopheles* females should suffer a fitness cost associated with infection, such as having shortened lifespans, producing fewer eggs (reduced fecundity), or producing fewer viable progeny (reduced fertility). However, given the obligatory reliance of *P. falciparum* on mosquitoes for transmission, there should be strong selective pressure on the parasite to not induce tradeoffs impacting survival, fecundity, or fertility. Indeed, a meta-analysis examining 11 published studies that assessed the effect of different *Plasmodium* strains on the survival of *Anopheles* species found that *P. falciparum* infection of its natural vectors (including *An. gambiae*, *An. stephensi*, and *An. funestus*) did not shorten mosquito lifespan, while infection of *An. stephensi* with rodent malaria strains (*Plasmodium berghei*, *Plasmodium yoelii nigeriensis*, and
Plasmodium chabaudi) induced longevity costs. Our own meta-analysis of 13 studies examining Plasmodium effects on Anopheles fecundity mirrors the survival data, showing that no reproductive cost as quantified by fecundity are incurred in natural Anopheles-Plasmodium interactions (see Chapter 1), though potential effects on fertility remain to be determined. No studies have tested the impact of P. falciparum infection on the fecundity of Anopheles species sampled from different geographic areas in parallel, and no studies have examined fitness costs in species of the Nyssorhynchus subgenus, leaving open the question of whether there is heterogeneity of fitness costs within anophelines, particularly among species that are not major vectors of this parasite.

In this study, we investigate whether an infectious P. falciparum blood meal induces fitness costs in anophelines sampled from different geographical locations: An. gambiae, the primary malaria vector in Africa (subgenus Cellia); An. stephensi, an important vector from the Indian subcontinent (subgenus Cellia); and An. albimanus, a minor vector from Central America (subgenus Nyssorhynchus). We find that while the fecundity of An. gambiae and An. stephensi is not affected by infection with P. falciparum, infected An. albimanus females produce significantly fewer eggs. We determine that in An. gambiae, this female tolerance to both laboratory and field isolates of P. falciparum is at least partially mediated by the 20E-sensitive female protein MISO. These studies demonstrate that a female protein that adapted in response to 20E transfer during mating confers tolerance to P. falciparum infection in the major malaria vector. As such, the evolution of this sexually-transferred 20E mating trait may have been favored or influenced by longstanding pressure from Plasmodium infection in a subset of Anopheles species.

RESULTS

Investigating the costs of Plasmodium falciparum infection on anopheline reproduction

In order to compare possible costs inflicted by P. falciparum on the reproductive fitness of Anopheles species, we fed An. gambiae, An. stephensi, and An. albimanus females on a blood
meal containing *P. falciparum* NF54 parasites, or a blood meal sampled from the same NF54 culture but heat-treated to inactivate the gametocytes in the culture and render the blood uninfected. These species originate from widespread geographical regions (Africa, India and Central America, respectively) and differ in their ability to synthesize 20E in their male accessory glands and transfer it to the female during mating. Forty-eight hours post-blood feeding (hpbf), female mosquitoes were collected and dissected to count the number of eggs developed per individual. Strikingly, *An. gambiae* and *An. stephensi* females produced the same number of eggs whether the bloodmeal was infectious or uninfected (*An. gambiae*: Student’s t test, two-tailed, t = 1.123, df = 159, p > 0.05; *An. stephensi*: Mann-Whitney test, two-tailed, Mann-Whitney U = 10391, p > 0.05), whereas *An. albimanus* females produced significantly fewer eggs when fed an infectious bloodmeal (Mann-Whitney test, two-tailed, Mann-Whitney U = 5088, p < 0.0001) (Figure 2.1). These data demonstrate that *An. albimanus* female mosquitoes suffer a fitness cost to their fecundity due to *P. falciparum* infection, while *An. gambiae* and *An. stephensi* do not.
Figure 2.1 | Diversity in fecundity costs associated with ingesting a *P. falciparum*-infected blood meal in anopheline vectors. Female mosquitoes of the African vector *An. gambiae*, the Indian vector *An. stephensi*, and the Central American vector *An. albimanus* were given a *P. falciparum* NF54-infected (darker-shaded dots) or uninfected (lighter-shaded dots) blood meal, and 48 hpf females were dissected to count the number of eggs developed in their ovaries. While *An. gambiae* and *An. stephensi* females fed infectious blood produce the same number of eggs as uninfected controls (*An. gambiae*: Student’s t test, two-tailed, $t = 1.123$, df = 159, $p > 0.05$; *An. stephensi*: Mann-Whitney test, two-tailed, Mann-Whitney $U = 10391$, $p > 0.05$), *An. albimanus* females fed an infectious culture produce significantly fewer eggs compared to females fed an uninfected bloodmeal (Mann-Whitney test, two-tailed, Mann-Whitney $U = 5088$, $p < 0.0001$). Data shows the results of 3 replicates for each species. The number indicated under each group is the number of mosquitoes sampled. Bars represent means with standard errors of the means.

**MISO mediates tolerance to *P. falciparum* infection**

Our data examining the effects of *P. falciparum* on the fecundity of different *Anopheles* species suggests that *An. gambiae* and *An. stephensi*, but not *An. albimanus*, have evolved mechanisms to reduce potential fitness costs associated with infection, a strategy known as
tolerance. To elucidate potential mechanisms underlying the difference in anopheline susceptibility to reproductive costs associated with *P. falciparum* infection, we focused on traits important for egg development that have diverged between New and Old World *Anopheles* species. *An. gambiae* and *An. stephensi* females receive 20E in the mating plug, while *An. albimanus* females do not \(^5\). In *An. gambiae*, this steroid hormone increases female fitness through interaction with the female protein MISO \(^5\). We therefore determined whether MISO produced following mating contributes to limiting the reproductive damage inflicted by *P. falciparum* in *An. gambiae* during infection.

One-day old females from the *An. gambiae* G3 colony (made of a mixture of *Anopheles gambiae sensu stricto* and *Anopheles coluzzii*) were injected with dsRNAs targeting either MISO (dsMISO) or the control GFP gene (dsGFP, or dsControl), mated, and fed on either a *P. falciparum* NF54-infected or uninfected blood meal. Two days post-blood feeding, they were collected and their ovaries were dissected to count the number of eggs developed per individual. While in these experiments we did not detect decreased fecundity in dsMISO relative to dsControl females fed on uninfected blood as previously reported \(^5\), we found that egg development was differentially affected by infection (one-way ANOVA, \(F = 4.63, p < 0.01\)) \((\text{Figure 2.2a})\). dsMISO females fed on infected blood produced significantly fewer eggs relative to their uninfected dsMISO counterparts (Tukey HSD test, \(p < 0.01\)), as well as relative to uninfected dsControl females (Tukey HSD test, \(p < 0.01\)) \((\text{Figure 2.2a})\). Infection intensity (Student’s t test on log\(_{10}\)-transformed data, two-tailed, \(t = 0.2674, df = 125, p > 0.05\)) and prevalence (Fisher’s exact test, two-sided, \(p > 0.05\)) were unaffected by MISO silencing \((\text{Figure 2.2b})\).
Figure 2.2 | MISO silencing reduces the fecundity of *P. falciparum*-infected females but does not impact parasite infection intensity and prevalence. Females from a laboratory colony of *An. gambiae* mosquitoes (G3) were silenced for MISO or the control gene GFP, mated, then fed on a *P. falciparum* NF54-infected or uninfected blood meal. (a) Egg development was significantly affected across three replicates (one-way ANOVA, $F = 4.63$, $p < 0.01$). While infection does not affect egg development in dsControl females, it significantly reduces the number of eggs in dsMISO females relative to infected dsControl and relative to uninfected dsMISO (Tukey’s multiple comparisons test, $p < 0.01$). (b) Across 5 replicates, infection intensity (Student’s t test on log_{10}-transformed data, two-tailed, $t = 0.2674$, df = 125, $p > 0.05$) and prevalence (Fisher’s exact test, two-sided, $p > 0.05$) was not impacted by MISO knock-down. $P(\%)$ represents the percent of females developing 1 or more oocyst. For a and b, lines represent means with standard error of the means (excluding the females that developed no oocysts in b), and N represents the number of females sampled.

The *An. gambiae* G3 colony was colonized from natural populations in The Gambia in 1975, while the *P. falciparum* NF54 strain has been in culture since the early 1980s. To ensure that the lack of a fitness cost in *An. gambiae* to *P. falciparum* infection and the potential role of MISO we recorded was not an artifact of using laboratory-adapted organisms but rather a phenotype relevant to natural Anopheles-Plasmodium dynamics, we conducted experiments in Burkina Faso using a recently colonized and periodically refreshed *An. gambiae* line of the M
molecular form, which is now classified as a separate sibling species *An. coluzzii*, and natural isolates of *P. falciparum* collected from gametocyte donors screened and recruited from villages neighboring Bobo-Dioulasso. One day-old females were silenced for MISO or the control LacZ gene using RNAi, crossed to males, and fed on *P. falciparum* infected blood. We selected a wide range of gametocytemias for the infection assays to test potential MISO effects across different intensities (Table 2.1). 7 days post-infection, the ovaries and midguts of females were dissected to count for eggs and oocysts, respectively, allowing us to collect paired egg-oocyst data for each individual. Contrary to our laboratory results, there was no significant difference between the two groups of females although the median number of eggs was lower in dsMISO (Mann Whitney test, $U = 6042$, $p > 0.05$) (Figure 2.3a). MISO knock-down did not affect oocyst intensities (Student’s t test on log$_{10}$-transformed data, $t = 0.4309$, df = 178, $p > 0.05$) or prevalence (Fisher’s exact test, two-sided, $p > 0.05$) (Figure 2.3b), confirming what was observed in laboratory infections.

However, when we analyzed the paired egg-oocyst data for dsMISO and dsControl female, we observed a striking effect. For this analysis, we implemented a generalized linear model (GLM) with a Poisson distribution and log link function to determine the effect of (1) replicate, (2) dsRNA treatment, (3) number of oocysts, and (4) the interaction of dsRNA treatment by number of oocysts factors on number of eggs developed. While the replicate ($\chi^2 = 5.621$, df = 2, $p > 0.05$), dsRNA treatment ($\chi^2 = 0.385$, df = 1, $p > 0.05$), and number of oocysts alone ($\chi^2 = 1.054$, df = 1, $p > 0.05$) did not show any effect, the interaction of dsRNA treatment by the number of oocysts significantly affected the number of eggs developed per female ($\chi^2 = 5.816$, df = 1, $p < 0.05$). Furthermore, the number of eggs developed was positively correlated to the number of oocysts developed in dsControl females (Spearman’s correlation, $\rho = 0.2288$, $p < 0.05$), whereas in dsMISO, this positive correlation was broken (Spearman’s correlation, $\rho = -0.0692$, $p > 0.05$) (Figure 2.3c).
Table 2.1 | Parameters for experimental infections of *An. coluzzii* with field isolates of *P. falciparum*

<table>
<thead>
<tr>
<th>Infection</th>
<th>Gametocytes (per µl of blood)</th>
<th>RNAi injection treatment</th>
<th>Number of mosquitoes</th>
<th>Oocyst intensity range</th>
<th>Oocyst intensity mean</th>
<th>Oocyst intensity median</th>
<th>Prevalence of infection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>104</td>
<td>dsControl</td>
<td>13</td>
<td>0-15</td>
<td>6.1</td>
<td>5.0</td>
<td>69.2</td>
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<tr>
<td></td>
<td></td>
<td>dsMISO</td>
<td>31</td>
<td>0-22</td>
<td>5.5</td>
<td>3.0</td>
<td>71.0</td>
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<tr>
<td>2</td>
<td>304</td>
<td>dsControl</td>
<td>34</td>
<td>0-178</td>
<td>30.9</td>
<td>16.0</td>
<td>76.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dsMISO</td>
<td>24</td>
<td>0-127</td>
<td>33.5</td>
<td>17.5</td>
<td>75.0</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>dsControl</td>
<td>19</td>
<td>0-22</td>
<td>4.2</td>
<td>2.0</td>
<td>73.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dsMISO</td>
<td>20</td>
<td>0-30</td>
<td>8.5</td>
<td>5.0</td>
<td>95.0</td>
</tr>
<tr>
<td>4</td>
<td>128</td>
<td>dsControl</td>
<td>22</td>
<td>0-87</td>
<td>26.3</td>
<td>27.0</td>
<td>90.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dsMISO</td>
<td>18</td>
<td>0-62</td>
<td>27.2</td>
<td>24.5</td>
<td>83.3</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>dsControl</td>
<td>36</td>
<td>0-17</td>
<td>5.2</td>
<td>3.5</td>
<td>83.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dsMISO</td>
<td>7</td>
<td>1-11</td>
<td>4.6</td>
<td>3.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Samples of *P. falciparum*-infected blood were collected from 5 gametocyte donors and fed to *An. coluzzii* mosquitoes silenced for *MISO* or a control gene. The number of gametocytes for each sample was counted per 1000 white blood cells, then converted to the number of gametocytes per µl of blood assuming a standard white blood cell count of 8000 cells per µl of blood. The outcome of infection was determined by counting the number of oocysts developed per female 7-8 days post-blood feeding.
To look more directly at the costs associated with developing *P. falciparum* infection at the oocyst level, we performed a logistic regression including only females that developed one or more oocysts and calculating their proportional likelihood of egg development across the observed range of oocyst intensities. Strikingly, while in the dsControl group the number of oocysts was not predictive of egg development and a female had an equal probability of failing to develop eggs across all oocyst intensities ($\chi^2 = 0.024, p > 0.05$) (Figure 2.3d, top panel), dsMISO females showed a greater likelihood of failing to develop eggs as they become more infected ($\chi^2 = 11.18, p < 0.001$) (Figure 2.3d, bottom panel). From this regression, we calculated that for every oocyst a dsMISO female develops, she is 3.6% more likely to fail developing eggs (Unit odds ratio, odds ratio = 1.036). These results taken together with our GLM and correlation studies reveal that MISO is a key factor mediating female *An. gambiae* tolerance to *P. falciparum* infection.
Figure 2.3 | *An. coluzzii* females depleted of MISO post-mating produce fewer eggs and have a greater likelihood of failing to develop eggs with increasing intensities of *P. falciparum* infection. *An. coluzzii* females were silenced for MISO or the control gene LacZ,
Figure 2.3 (Continued) mated, and fed on a wide range of gametocytemias (see Table 2.1). (a) Infected dsMISO females do not produce fewer eggs relative to infected controls (Mann Whitney test, $U = 6042, p > 0.05$). Lines represent medians, N represents the number of females sampled, and P(%) represents the percent of females developing 1 or more egg. (b) MISO knock-down does not affect oocyst intensities (Student’s t test on log$_{10}$-transformed data, $t = 0.4309, df = 178, p > 0.05$) or prevalence (Fisher’s exact test, two-sided, $p > 0.05$). Lines represent means with standard error of the means excluding the females that developed no oocysts, N represents the number of females sampled, and P(%) represents the percent of females developing 1 or more oocyst. (c) The number of eggs and oocysts developed per female is positively correlated in dsControl (Spearman’s correlation, $\rho = 0.2288, p < 0.05$), but this correlation is broken in dsMISO (Spearman’s correlation, $\rho = -0.0692, p > 0.05$). Each dot represents paired egg-oocyst data for one female. Lines represent linear regressions of data points for dsControl and dsMISO. (d) Logistic regressions calculating the proportional likelihood of dsControl and dsMISO females failing to develop eggs with increasing $P. falciparum$ oocyst intensities show that in dsControl group, the number of oocysts is not predictive of egg development: a female has an equal probability of failing to develop eggs across all oocyst intensities ($\chi^2 = 0.024, p > 0.05$) (top panel). In dsMISO females, the number of oocysts developed is highly predictive of egg development: dsMISO females have a greater likelihood of failing to develop eggs as they become more infected ($\chi^2 = 11.18, p < 0.001$) (bottom panel).

**Correlation networks built from transcriptional data offer clues to the molecular pathways MISO may be acting in**

How can a mating-induced protein expressed in the atrium affect *An. gambiae* tolerance to both laboratory and field $P. falciparum$ strains? Little evidence can be gathered from its protein sequence and structure, as MISO is a small, 152 amino acid glycine-rich protein with a signal peptide but no known functional domains. Furthermore, attempts to find potential interacting partners via co-immunoprecipitation assays were unsuccessful because our polyclonal anti-MISO antibody, while suitable for Western and immunofluorescence (IFA) assays, failed to efficiently immunoprecipitate this protein in vivo. We therefore decided to use an *in silico* approach to determine what pathways or processes MISO may be involved in by inputting 31 2-color microarray datasets (both published and unpublished) from 6 experimental groups into an application developed in ShinyR by Victoria Ingham and colleagues at LSTM (Ingham et al., manuscript in preparation). The application can identify co-regulated transcripts and generate correlation networks of a particular gene by producing a pairwise correlation matrix for each transcript using Pearson’s correlation coefficients. Identification of these co-regulated transcripts
can then aid in determining the putative pathways or processes involving a particular gene. Transcriptional data sets included can be found in Table S1 and were selected to represent samples that 1) are sensitive to 20E \(^6\) and/or ecdysone receptor (EcR) knock-down (unpublished) given that MISO expression is regulated by 20E through EcR following mating \(^5\); 2) examine gene expression following blood feeding \(^36,37\), based on MISO’s effect on \(P. falciparum\); and 3) are related to stress and detoxification responses \(^38,39\), as MISO is important to maintain fitness following parasite infection.

Using an arbitrary but stringent cut-off correlation value of 0.7, a list of 22 co-regulated genes was generated (Table 2.2) and mapped out to examine the strength of correlation (Figure 2.4). Interestingly, this list included genes encoding two hormonal factors that play a role in ecdysis: Hormone Receptor 4 (HR4, AGAP004693) and Ecdysis Triggering Hormone (ETH, AGAP007062) (Table 2.2 and Figure 2.4, in red). In Drosophila, HR4 is a nuclear receptor involved in larval molting and functions to control ecdysone signaling \(^40\). During larval molting, this receptor translocates from the cytoplasm to the nucleus of Drosophila endocrine cells in the prothoracic gland to repress the expression of the cytochrome P450 Cyp6t3 that functions in the ecdysone biosynthesis pathway \(^40\). This repression in turn reduces ecdysone production and eventually stops the ecdysone pulse, terminating ecdysis \(^40\). The function of HR4 in Anopheles has yet to be investigated. ETH is a key molting neuropeptide hormone produced and released from secretory Inka cells associated with the trachea \(^41\). During each molt as insects move to the next larval stage, a new tracheal network filled with molting fluid is built to accommodate for the larger size of the next stage. At the completion of the molt, ETH is released, which stimulates the new trachea to fill with air, enabling shedding of the old tracheal cuticle and allowing the larvae to complete ecdysis (reviewed in \(^42\)). Production and release of ETH in Inka cells is regulated by the peak and decrease of 20E, respectively \(^43,44\), and this endocrine signaling molecule is remarkably conserved in insects \(^45\), including in \(An. gambiae\) \(^46\). A recent publication found that in Drosophila, ETH continues to be produced in Inka cells located on the abdominal trachea in adult females,
and that deficient ETH signaling in adulthood results in reduced egg production and lipid accumulation in oocytes. Furthermore, the authors report that as is the case in larval stages, ETH regulation in adulthood is mediated by 20E, as 20E injection in adult females increased transcription of ETH and its receptor ETHR.

### Table 2.2 | MISO co-regulated gene set from transcriptional analysis

<table>
<thead>
<tr>
<th>Correlation</th>
<th>Gene ID</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AGAP002620</td>
<td>mating-induced stimulator of oogenesis MISO</td>
</tr>
<tr>
<td>0.913548365</td>
<td>AGAP001303</td>
<td>chemosensory protein 6</td>
</tr>
<tr>
<td>0.912247054</td>
<td>AGAP006426</td>
<td>cyanogenic beta-glucosidase</td>
</tr>
<tr>
<td>0.9120568</td>
<td>AGAP007042</td>
<td>cuticular protein RR-1 family 62</td>
</tr>
<tr>
<td>0.901728769</td>
<td>AGAP009402</td>
<td>odorant-binding protein 43</td>
</tr>
<tr>
<td>0.860798385</td>
<td>AGAP005724</td>
<td>lipoma HMGIC fusion partner-like protein 4</td>
</tr>
<tr>
<td>0.846134512</td>
<td>AGAP010706</td>
<td></td>
</tr>
<tr>
<td>0.823199437</td>
<td>AGAP006118</td>
<td></td>
</tr>
<tr>
<td>0.814759458</td>
<td>AGAP006501</td>
<td></td>
</tr>
<tr>
<td>0.790438763</td>
<td>AGAP012530</td>
<td></td>
</tr>
<tr>
<td>0.782720129</td>
<td>AGAP007602</td>
<td>ecdysis-triggering hormone</td>
</tr>
<tr>
<td>0.763385312</td>
<td>AGAP007250</td>
<td></td>
</tr>
<tr>
<td>0.749677555</td>
<td>AGAP009833</td>
<td>voltage-dependent anion-selective channel protein 2</td>
</tr>
<tr>
<td>0.739861192</td>
<td>AGAP002205</td>
<td>CYP325C2</td>
</tr>
<tr>
<td>0.739160884</td>
<td>AGAP010255</td>
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<tr>
<td>0.728047153</td>
<td>AGAP003468</td>
<td>osiris 17</td>
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<td>0.724580674</td>
<td>AGAP000821</td>
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<td>0.722656883</td>
<td>AGAP010022</td>
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<tr>
<td>0.720841196</td>
<td>AGAP003375</td>
<td>cuticular protein RR-2 family 114</td>
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<td>0.715235523</td>
<td>AGAP009102</td>
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</tr>
<tr>
<td>0.710421884</td>
<td>AGAP004693</td>
<td>Hormone Receptor 4</td>
</tr>
<tr>
<td>0.710413616</td>
<td>AGAP006502</td>
<td></td>
</tr>
</tbody>
</table>

Gene names were collected from VectorBase community annotations.

In light of ETH being identified as a co-regulated gene, we were interested to find 2 genes in the list that are annotated as cuticular proteins (AGAP003375, AGAP007042) (Table 2.2 and Figure 2.4, in green), as the tracheal cuticle is composed of cuticular proteins, and at each molt these proteins would likely be synthesized to generate new, larger trachea capable of oxygenating
the next larval stage. Among the other genes was the chemosensory protein AGAP001303, the most strongly co-regulated gene with MISO. Chemosensory proteins are small soluble proteins found only in arthropods \(^{49}\) that were initially implicated in pheromone sensing and binding of volatile compounds. Recent studies have shown they can function in roles beyond chemosensation \(^{50}\), including a report in the honeybee *Apis mellifera* where a chemosensory protein transcribed in ovaries and embryos was shown to be important for normal embryonic integument formation, as silencing of this gene prevented eggs from hatching \(^{51}\).

**Figure 2.4 | Putative co-regulated transcripts of MISO based on *in silico* analysis of microarray datasets.** 31 2-color microarray datasets from 6 studies were inputted into an application developed in ShinyR \(^{34,35}\) (Ingham *et al.*, manuscript in preparation), and a pairwise correlation matrix for each transcript using Pearson’s correlation coefficients was developed for MISO. The resulting correlation network found that MISO is co-regulated with 2 20E-sensitive hormonal factors ETH (AGAP007062) and HR4 (AGAP004693) (in red), as well as 2 cuticular proteins (AGAP007042 and AGAP003375) (in green). The strength of the correlation is indicated by both branch thickness and length.
MISO is produced in the ovaries and associated trachea in a 20E-dependent manner following blood feeding

We decided to follow up on the results of the *in silico* analysis suggesting MISO is co-regulated with ETH, and we determined if MISO is also produced in the trachea of *An. gambiae* females, particularly the trachea associated with the ovaries given the effect of MISO knock-down on egg development. Our published transcriptional analyses found MISO expression at very high levels in the atrium post-mating, but did not detect transcripts in the ovaries or the carcass post-mating or blood feeding \(^5\); however, these studies did not look at earlier timepoints in adulthood. We decided to perform a more thorough analysis of the ovaries and did transcriptional profiling of this gene from early stages of adult life. We extracted RNA from the ovaries of females collected at 1, 4, and 5 days post-eclosion from the pupal stage, as well as at 3, 24, and 48 hpbf. Surprisingly, qRT-PCR of MISO revealed that this gene is highly expressed in ovaries of 1 day old females, a time when these tissues are still immature and are largely made up of tracheal-like tissue (*Figure 2.5a*). Expression decreased from immature to mature ovaries during adulthood, and was not induced by blood feeding and was even further repressed in mated blood-fed females (Multiple t tests at 24 hpbf with Holm-Sidak correction for multiple comparisons; virgin females: \(t\) ratio = 2.54, df = 4, adjusted \(p > 0.05\); mated females: \(t\) ratio = 3.49, df = 4, adjusted \(p < 0.05\)), explaining why MISO expression in the ovaries was not detected in previous studies (*Figure 2.5a*).

We analyzed protein levels by Western blot and found that consistent with the transcriptional data, MISO is expressed at high levels in 1 day-old immature ovaries (a band at approximately 17 kDa in size), which contain both ovarian and associated tracheal tissues, and that levels decrease once these tissues mature (0 and 6 hours post-blood feeding) (*Figure 2.5b*). However, while at 24 hpbf we see decreased transcript levels, we detect higher levels – or often a crisper band – of MISO at this time-point, with peak levels at 36 hpbf (*Figure 2.5b* and Figure
Furthermore, there is no detectable difference in the levels produced in virgin and mated females post-blood feeding (Figure S1).

To investigate whether MISO production in the ovaries/trachea is regulated by 20E produced following blood feeding as it is in the atrium following mating, we first injected physiological levels of this steroid hormone into the thorax of adult An. gambiae females, and 24 hours post-injection (hpi) we dissected and collected ovaries and associated trachea and blotted for MISO. We found that while the blood-fed positive control samples had high levels of MISO 36 hpf, 20E-injected unfed ovaries/trachea did not show increased levels relative to ethanol-injected controls (Figure 2.5c). However, when females were injected with the 20E-inactivating oxidase E22O and blood fed 8-10 hours later, we detected significantly lower levels of MISO in these tissues at 24 and 36 hpf (Figure 2.5d). These data demonstrate that MISO production in the ovaries and/or trachea is regulated by 20E but that this signal is blood feeding-dependent, suggesting expression of this protein is reliant on additional signaling cascades induced by blood feeding.
Figure 2.5 | Transcriptional and protein analysis of *An. gambiae* ovaries demonstrates MISO is produced in the ovaries in 1 day old females and post-blood feeding in a 20E-dependent manner. (a) qRT-PCR on 10-15 ovaries/trachea of females collected at 1, 4, and 5 (0 hpbf) days post-eclosion and at 3, 24, and 48 hpbf show that MISO is transcribed at high levels in 1 day old females, but that transcript levels decrease in later adulthood. Blood feeding decreases transcript levels in both virgin and mated females. MISO transcript levels were normalized to the housekeeping gene *RPL19* (AGAP004422) using the ∆C_T method. Data is the result of 3 replicates, error bars represent standard errors of the means. (b) Protein was extracted from 10-15 ovaries of 1 day old females, as well as non-blood-fed (5 days old), 6, 24, 36, and 48 hpbf females and probed for MISO as well as for actin (as a loading control). MISO is 15 kDa, but runs at a size of approximately 17 kDa and is found at high levels in 1 day old ovaries/trachea, decreases in later stages, but increases again at 36 hpbf. (c) MISO is present in the ovaries of females 36 hpbf, but this production is not mediated by 20E alone, as 20E-injected females did not produce high levels of the protein 24 hpi. (d) Injection of the 20E-inactivating oxidase E22O reduces the level of MISO in ovaries post-blood feeding. A 20 kDa band is consistently detected in ovarian samples probed for MISO. Attempts to deglycosylate and dephosphorylate this protein did not induce a size shift in the band ([Supplementary Methods](#) and [Figure S2a](#) and [Figure S2b](#)), suggesting this is a non-specific band detected by our polyclonal antibody.
We next performed IFAs to assess whether MISO could be detected in the trachea, the ovaries, or both tissues using the same polyclonal MISO antibody used in Western blot assays. MISO was detectable in a diffuse pattern in the trachea of non-blood-fed females (Figure 2.6, left panel), but upon blood feeding, it localized in a more punctate pattern in the smaller tracheal segments and was no longer present in the major branches, particularly at 24 hpbf (Figure 2.6, right panel). In the ovaries, MISO was found at 6 hpbf in a mesh-like structure enveloping the oocytes which will eventually develop into eggs. This mesh-like pattern around the oocyte notably excludes the nurse cells that are factories for nucleic acids that will be transferred into the oocyte as it grows into an egg (Figure 2.6, central panel). To determine the specificity of this signal, we incubated MISO polyclonal antibody with the corresponding immunizing peptide at a 200-fold molar excess, and this antibody sample was used to stain ovaries in parallel with ovaries stained with MISO antibody alone. This peptide competition assay (PCA) demonstrated the signal in the ovaries and trachea is specific, as it disappeared in ovaries stained with MISO antibody incubated with the immunizing peptide (Figure 2.6, panels labeled with “+” to indicate antibody incubation with the immunizing peptide). Beyond the previously identified role of MISO in transducing sexually-transferred 20E in the atrium to enable normal yolk accumulation in the eggs, the presence of MISO protein in 1 day-old immature ovaries and post-blood feeding, its localization encapsulating oocytes in ovarian follicles post-blood feeding, and its sensitivity to 20E signaling from blood feeding suggest this protein plays additional roles in the ovaries. It could potentially function to ensure proper, timely egg development at the cellular level in what is a very tightly regulated process. This hypothesis is supported by MISO’s putative co-regulation with ETH, ETH’s role in signaling to ensure normal Drosophila egg development, and the fact that MISO protein is present at high levels in the trachea, the tissue sensitive to ETH signaling.
Figure 2.6 | MISO localizes to the trachea and in the oocyte post-blood feeding. Ovaries were dissected from non-blood-fed (left panel), 6 hpf (center panel), and 24 hpf (right panel) females. These tissues were fixed and incubated with MISO antibody (-) or MISO antibody with the immunizing peptide (+). Non-blood-fed females have diffuse MISO signal in their trachea, which disappears in the ovaries incubated with antibody from the PCA (left panel). At 6 hpf, the signal in the trachea begins to localize to the finer tracheal branches, and can also be seen in a mesh-like pattern surrounding the oocytes within ovarian follicles (center panel, -). Again, this signal disappears in the PCA samples (center panel, +). At 24 hpf, MISO localizes in patches in the finest tracheal branches (right panel, -), and this bright signal disappears in PCA ovaries (right panel, +). Images are from maximum projections of Z-stacks. Scale bar = 50 µm.
Figure 2.6 (Continued)
Given our findings that MISO transcripts and protein are produced in the ovaries and trachea, we next sought to determine whether the effects of MISO on egg development are due to its expression in ovarian/tracheal tissues rather than or in addition to a possible role in the atrium after mating. qRT-PCR analysis on MISO expression in the atria of dsMISO females relative to dsControl consistently showed more than 80% knock-down efficiency across multiple experiments (Figure S3a). MISO transcripts were also significantly reduced in the ovaries of dsMISO females relative to controls (Two-way ANOVA, dsRNA: F = 58.13, df = 1, p < 0.0001) (Figure S3b). However, while Western blot analysis is in agreement with the MISO transcriptional data in the atrium and shows an absence of MISO post-mating, we were not able to consistently detect reduced levels of this protein in the ovaries of dsMISO females post-blood feeding over many experiments (Figure S3c), suggesting silencing in this tissue is not always effective. Therefore, we cannot state with full confidence that the effects we observed in P. falciparum infections are due to MISO silencing in the ovaries and associated trachea, but could instead result from efficient knock-down in the atrium post-mating.

MISO depletion dampens the transcription of hypoxia-induced factors post-blood feeding and post-mating

As previously mentioned, the function of the trachea has been associated with ecdysis: in larval stages the signal to molt occurs in part because the larvae grow to a size that the fixed tracheal system can no longer provision with oxygen, leading to low oxygen (hypoxic) conditions and stimulating the release of 20E to trigger ecdysis. Consistently, tracheal development and molting are regulated by hypoxia-inducible factors, including the aryl hydrocarbon receptor nuclear translocator (ARNT), also called hypoxia-inducible transcription factor beta (HIFβ), as well as by HIFα and the prolyl hydroxylase fatiga. Hypoxia-induced signaling is mediated by the heterodimer HIFα/β which translocates to the nucleus under low oxygen conditions to induce transcription of hypoxia-responsive genes. Fatiga hydroxylates HIFα under conditions of
normoxia to target it for degradation, while HIFβ is constitutively expressed and remains stable at the protein level in conditions of normoxia and hypoxia\(^\text{57}\). HIFβ mutant embryos have branching defects in their trachea\(^\text{55}\); HIFα localizes to the larval trachea and mutants cannot continue their larval development under hypoxic stress\(^\text{56}\); and fatiga mutants have defective trachea that fail to gas-fill\(^\text{56}\). Furthermore, a recent study demonstrated that gut hypoxia induces 20E pulses and transcription of HIFα and fatiga in larvae\(^\text{58}\), and that inhibiting HIFα function in adult stages leads to aberrant Lp expression and lipid storage in the fat body\(^\text{59}\).

Given the fact that MISO is regulated by 20E both post-mating and post-blood feeding, that it is detected in the ovaries and trachea post-blood feeding, and that depletion of this protein during \textit{P. falciparum} infections leads to impaired egg development, we investigated whether \textit{MISO} may be regulated by hypoxia. To this end, we first analyzed the promoter region of this gene using Genomatix Matinspector software\(^\text{60,61}\). In this analysis, we uncovered a number of binding sites for AP-1 transcription factors, proteins that are involved in apoptosis, as well as in cell proliferation and differentiation. We conducted several studies to determine whether \textit{MISO} functions within the JNK pathway, which signals through AP-1 transcription factors, but this work did not produce conclusive results (a summary of these studies can be found in Appendix B). Genomatix analysis also revealed 7 putative binding sites for HIF transcription factors (Table S2). We then determined whether hypoxia-induced signaling is affected by \textit{MISO}-silencing. ds\textit{MISO} and ds\textit{Control} females were mated and blood fed, and pools of ovaries were collected post-blood feeding. qRT-PCR on these samples showed that \textit{HIFβ} (AGAP028645) expression was significantly induced from 3 to 48 hpbf in control females, (one-way ANOVA, \(F = 4.764, p > 0.05\); Tukey HSD test 3 vs. 48 hpbf, \(p < 0.05\)), while this induction was not observed after \textit{MISO} silencing (one-way ANOVA, \(F = 5.212, p < 0.05\); Tukey HSD test 3 vs. 48 hpbf, \(p > 0.05\)) (Figure 2.7a). \textit{Fatiga} (AGAP003523) was significantly induced post-blood feeding in both \textit{dsControl} (one-way ANOVA, \(F = 7.962, p < 0.05\); Tukey HSD test 3 vs. 48 hpbf, \(p < 0.05\); Tukey HSD test 24 vs. 48 hpbf, \(p < 0.05\)) and \textit{dsMISO} (one-way ANOVA, \(F = 14.06, p < 0.01\); Tukey HSD test 3 vs. 48 hpbf, \(p < 0.01\); Tukey HSD test
24 vs. 48 hpbf, \( p < 0.05 \)), though the data shows a trend that the fold induction at 48 hpbf relative to non-blood-fed females could be lower in dsMISO (Figure 2.7b). \( HIF\alpha (\text{AGAP002942}) \) was expressed at levels below the detection limit of our assay in all samples. We also examined the effect of mating on \( HIF\beta \) and \( fatiga \) expression in the reproductive tissues (atria and ovaries together), and found that time post-mating significantly affects both these genes (two-way ANOVA, time post-mating – \( HIF\beta \): \( F = 7.69, \text{df} = 2, p < 0.01 \); \( fatiga \): \( F = 6.36, \text{df} = 2, p < 0.05 \)). Moreover, \( MISO \) silencing significantly impacted \( HIF\beta \) (Two-way ANOVA – dsRNA: \( F = 12.04, \text{df} = 1, p < 0.01 \)) (Figure 2.7c) and \( fatiga \) expression (two-way ANOVA – dsRNA: \( F = 14.52, \text{df} = 1, p < 0.01 \)) across all timepoints (Figure 2.7d). These results are consistent with what we observed in the ovaries post-blood feeding, though \( fatiga \) expression seems more susceptible to \( MISO \) silencing in atria and ovaries post-mating. All together, these results suggest that MISO is involved in hypoxia signaling, as its silencing negatively affects the blood feeding- and mating-induced expression of two key components of this signaling pathway.
Figure 2.7 | HIF signaling is induced in An. gambiae reproductive tissues post-blood feeding and post-mating and is dampened in MISO-depleted females. qRT-PCR analysis using primers targeting An. gambiae HIFβ (AGAP028645) and fatiga (AGAP003523) was performed on pools of ovaries post-blood feeding (a and b) and pools of atria and ovaries post-mating (c and d). (a and b) ∆C_T values for each injection treatment post-blood feeding were normalized to the ∆C_T of non-blood fed females of each injection treatment. (a) HIFβ expression is significantly induced in dsControl ovaries from 3 to 48 hpbf (one-way ANOVA, F = 4.764, p = 0.0577; Tukey HSD test, p < 0.05; all other comparisons Tukey HSD test, p > 0.05), but not in dsMISO ovaries (one-way ANOVA, F = 5.212, p = 0.0488; all comparisons Tukey HSD test, p > 0.05). (b) Fatiga expression is significantly increased by blood feeding in both dsControl (one-way ANOVA, F = 7.962, p < 0.05; Tukey HSD test 3 vs. 24, p > 0.05; Tukey HSD test 3 vs. 48 hpbf, p < 0.05; Tukey HSD test 24 vs. 48 hpbf, p < 0.05) and dsMISO ovaries (one-way ANOVA, F = 14.06, p < 0.01; Tukey HSD test 3 vs. 24 hpbf, p > 0.05; Tukey HSD test 3 vs. 48 hpbf, p < 0.01; Tukey HSD test 24 vs. 48 hpbf, p < 0.05). (c) Two-way ANOVA analysis demonstrates HIFβ
Figure 2.7 (Continued) expression is affected by time post-mating (time post-mating: $F = 7.69$, $df = 2$, $p < 0.01$) and reduced in dsMISO females ($dsRNA$: $F = 12.04$, $df = 1$, $p < 0.01$), but the interaction of dsRNA and time post-mating is not significant ($F = 0.1554$, $df = 2$, $p > 0.05$). (d) Two-way ANOVA analysis demonstrates fatiga expression is affected by time post-mating (time post-mating: $F = 6.36$, $df = 2$, $p < 0.05$) and reduced in dsMISO females ($dsRNA$: $F = 14.52$, $df = 1$, $p < 0.01$), but the interaction of dsRNA and time post-mating is not significant ($F = 0.2658$, $df = 2$, $p > 0.05$). 3 replicates of each timecourse were collected.

Levels of apoptosis are not altered in the ovaries of MISO-depleted females following *P. falciparum* infection

Our laboratory and field results show that in conditions of *P. falciparum* infection, *An. gambiae* females lacking MISO produce fewer eggs. We also show that MISO is present in the ovaries and trachea post-blood feeding, and that silencing of this gene dampens components of HIF-signaling post-mating and post-blood feeding. We next set out to determine the mechanisms by which MISO affects egg development in females infected with *P. falciparum*. Is the reduced fecundity due to stalled development, or is it caused by an active process of cell death of ovarian follicles? Under conditions of nutritional stress in *Drosophila*, ecdysone production was shown to increase and induce ovarian apoptosis, leading to reduced egg chamber numbers $^{62}$. Based on this finding, we investigated whether decreased egg development in MISO-depleted *An. gambiae* infected with *P. falciparum* was due to increased apoptosis in ovarian follicles. Females injected with dsRNA targeting either MISO or GFP were mated and fed on blood infected with *P. falciparum*. Ovaries of these females were dissected at 18 hpbf and stained to detect apoptosis by TUNEL assay. While some ovary pairs collected from dsMISO females (*Figure 2.8a*, top panels) showed increased TUNEL staining in primary ovarian follicles compared to dsGFP females (*Figure 2.8a*, bottom panels), the overall proportion of primary follicles stained per ovary was not significantly different between the groups (Mann-Whitney test, two-tailed, Mann-Whitney $U = 399$, $p > 0.05$) (*Figure 2.8b*).

We also performed a plate-based caspase activity assay to detect potential differences in apoptosis in the ovaries. MISO-silenced females were fed on *P. falciparum* and ovaries were
dissected post-blood feeding and assayed for caspase-3/7 enzymatic activity. While caspase-3/7 activity significantly increased post-blood feeding in both groups (Two-way ANOVA, F = 65.78, df = 2, p < 0.0001), we detected no difference between infected dsMISO and controls (Two-way ANOVA, F = 0.6819, df = 1, p > 0.05) (Figure 2.8c), suggesting that the decrease in the number of eggs produced by dsMISO females infected with *P. falciparum* is not mediated by increased apoptosis in the ovarian follicles and may instead be due to developmental or growth defects.

![Image](image_url)

**Figure 2.8 | MISO silencing does not impact apoptosis levels in the ovaries of females infected with *P. falciparum*.** Females were injected with dsRNA targeting MISO or the control gene GFP, mated, then fed on a *P. falciparum*-infected blood meal. (a) 18 hpbf, ovaries were
Figure 2.8 (Continued) stained to detect apoptosis using a TUNEL assay. (b) The number of apoptotic primary follicles was counted and divided by the total number of primary follicles to determine the proportion of primary follicles undergoing apoptosis at 18 hpbf. Over three replicates, no statistical difference in the proportion of apoptotic primary follicles was detected between dsControl and dsMISO (Mann-Whitney test, two-tailed, Mann-Whitney U = 399, p > 0.05). N represents the number of females assayed per group. (c) To determine overall level of caspase activity in the ovaries as a proxy for apoptosis in dsControl and dsMISO females, pools containing ovaries of each injection group were collected at 3, 12, and 24 hpbf. The level of caspase 3/7 activity was determined in each pool. While the level of caspase activity increases post-blood feed (Two-way ANOVA, F = 65.78, df = 2, p < 0.0001), there is no difference between dsMISO and dsGFP females (Two-way ANOVA, F = 0.6819, df = 1, p > 0.05).

DISCUSSION

Our study shows that An. gambiae females do not suffer a reproductive cost to P. falciparum infection, measured as the number of eggs developed after blood feeding, and that this tolerance to infection is conferred in part by the female protein MISO, as silencing of this gene reduced the fecundity of P. falciparum-infected females. It remains to be determined whether Plasmodium infections affect other processes during oogenesis that may not impact egg numbers but may impair the fitness of developed eggs, thereby reducing female fertility. Previously, we had reported that expression of MISO was specifically induced in the female atrium by sexual transfer of 20E. We showed that MISO forms a complex with 20E, increases the expression of lipid transporters, enables normal vitellogenesis in the ovaries, and increases female fecundity. We now demonstrate that MISO is also sensitive to the production of 20E post-blood feeding, as we detect high levels of this protein in the ovaries and trachea of blood-fed females. MISO was also detected in immature adult ovaries post-pupal eclosion, possibly due to 20E produced in pupae that could mediate pupal to adult ecdysis, as has been demonstrated in the insect Manduca sexta. In An. gambiae ovaries post-blood feeding, its localization is within the ovarian follicle, specifically encapsulating the oocytes, the part of the ovarian follicle that will develop into an egg. We also find MISO at high levels in associated tracheal tissues whose function is to oxygenate the ovaries. Moreover, the MISO regulatory region contains multiple binding sites for HIF transcription factors, and correlation network analysis from multiple microarray datasets show this
gene may be co-regulated with the tracheal hormone ETH, involved in the regulation of molting. No definitive connection has been made yet between HIF and ETH-mediated signaling; however, the role of both of these pathways in tracheal development, molting, and tissue oxygenation, combined with their relationship to 20E signaling, suggests these processes are linked. Therefore, we speculate that MISO may function within hypoxia-induced and ETH signaling pathways to regulate ovarian tissue growth and egg development.

Our previous study showed that MISO silencing reduced the number of eggs developed per female relative to controls \(^5\); however, in our laboratory infection experiments, we did not see a similar decrease in fecundity in dsMISO females fed on uninfected blood relative to uninfected dsControl females (Figure 2.2a). This difference could stem from a few sources. First, the G3 colony is a hybrid of the two sibling species An. gambiae s.s. and An. coluzzii, and we have recently shown that in natural populations of these two species, the level of MISO induction post-mating varies, with An. coluzzii having higher fold induction from virgin to mated, as well as higher overall expression levels post-mating compared to An. gambiae s.s \(^64\). The original MISO characterization experiments \(^5\) were conducted on a different G3 colony than the one used in this study, and it is possible the ratio of An. gambiae s.s. to An. coluzzii differed in these two groups and resulted in variation in MISO-related phenotypes. Furthermore, certain environmental factors can decrease egg development in insects, including nutrient deprivation during larval development \(^65,66\) and thermal stress \(^67,68\), and in Drosophila, the response to some of these stressors is to increase 20E levels \(^69,70\). Therefore, different breeding conditions of the two G3 colonies used in these separate studies could also explain the variation in susceptibility to MISO silencing.

While evidence from the literature points to a role for ETH in lipid accumulation in the oocytes and in egg development \(^47\), no studies have examined the function of ETH in egg development, or indeed, in any capacity in An. gambiae. Our in silico analysis of microarray datasets reveals a potential co-regulation of MISO and ETH, but future studies will need to
validate these results. Transcripts of the putative *An. gambiae* ETH and *ETHR* (AGAP002881) were below the level of detection by qRT-PCR analysis in the reproductive tissues of females post-mating and post-blood feeding, complicating our ability to determine if MISO silencing affects expression of these genes. Future work will use ETH silencing experiments to elucidate a potential role for this neuropeptide hormone on egg development in *An. gambiae* and determine whether it functions with MISO. Moreover, given our finding that MISO may also be co-regulated with the nuclear hormone receptor *HR4* which functions to terminate ecdysone pulses during larval development \(^{40}\), future studies will also investigate the potential link between HR4, egg development, and MISO by silencing HR4.

While we have yet to demonstrate the occurrence of hypoxia in ovaries and its role in egg development, our data showing that *HIFβ* and *fatiga* expression is stimulated by blood feeding and mating is the first report of hypoxia-induced signaling in these biological processes in *Anopheles* mosquitoes and indeed, in blood feeding insects. In *Drosophila*, this signaling pathway has been implicated in egg development through its role in border cell migration \(^{71}\) (a process required for the formation of the micropyle, a structure that enables fertilization of the egg \(^{72}\)) and in follicular epithelial cell morphogenesis \(^{73}\), strengthening the possibility that hypoxia plays a developmental role in anopheline egg development as well. Furthermore, the fact that MISO silencing affects expression of *HIFβ* and *fatiga*, particularly in reproductive tissues post-mating, implicates HIF in 20E-mediated signaling. In support of this hypothesis, a recent study found that peaks of hypoxia in the *Aedes aegypti* larval gut generated by the microbiome stimulate 20E production and enable larval development through successive molting stages \(^{58}\). Future work will focus on characterizing the role of hypoxia-induced signaling in egg development as well as a potential mechanistic link with MISO through the use of a hypoxia signaling antagonist (PX-478) and agonist (FG-4592), and through knock-down studies of HIF transcription factors. Furthermore, additional studies will need to be done to understand why HIF\(\alpha\) expression was below the level of detection in our assays and whether its transcription is regulated in the case of
blood feeding and/or mating in \textit{An. gambiae}, as it is usually more transcriptionally responsive than HIF$\beta$\textsuperscript{57}. Indeed, our failure to detect \textit{HIF}a by qRT-PCR could be due to poor annotation of this gene in \textit{An. gambiae}, as \textit{AGAP002942} is listed as the \textit{HIF}a/sima ortholog in FlyBase (FB2018_02)\textsuperscript{74}, but VectorBase\textsuperscript{48} annotation of this gene indicates no orthologues in \textit{Drosophila}. As a result, further work must be done to determine whether \textit{AGAP002942} is truly the \textit{An. gambiae} \textit{HIF}a.

How could blood feeding trigger signaling via hypoxia-induced transcription factors? The digestion of the blood meal could generate molecules that activate hypoxia-induced responses. For example, \textit{An. gambiae} mosquitoes produce ROS, including hydrogen peroxide (H$_2$O$_2$) and superoxide (O$_2^-$), post-blood feeding\textsuperscript{75}, and these molecules could function to activate signaling through HIFa/β. Studies in mammalian cells found that ROS released from mitochondria activated hypoxia transcriptional responses by inhibiting fatigue\textsuperscript{76,77}. Another possibility is that the significant ROS production\textsuperscript{78} caused by the expansion of the midgut microbiome that occurs in \textit{An. gambiae} post-blood feeding\textsuperscript{79,80} could activate hypoxia-mediated signaling and stimulate the production of 20E hormone as has been recently demonstrated in \textit{Ae. aegypti} larvae\textsuperscript{58}. There is less evidence to understand how or for what purpose HIF-signaling may be regulated in the reproductive tissues post-mating. Following mating, the atria of \textit{An. gambiae} undergo drastic structural changes due to the transfer of the plug\textsuperscript{13}. In particular, the size of the plug, and likely also the force of its transfer, cause distention in the atrium\textsuperscript{13}. Given that the luminal side of the atrium is lined with cuticle, it is possible that distention of this cuticle elicits the same signaling cascades stimulated by the trachea in larvae during molting. Indeed, the tracheal cuticle stretches as the larval body grows to proportions that the fixed tracheal system can no longer adequately oxygenate, resulting in hypoxia and stimulating ecdysis\textsuperscript{54,81}. Further studies need to be done to examine a possible role of the hypoxic response post-mating and post-blood feeding, including silencing and/or antagonism of HIF factors.
Although we have evidence that MISO may function within HIF-signaling cascades, its role within this pathway remains elusive. However, the timing of peak MISO at the protein level relative to the peak of 20E post-mating and post-blood feeding can offer clues as to its function. It appears that following both mating and blood feeding, the peaks of MISO levels are concomitant with a decrease in 20E titers. In the female atrium post-mating, male 20E levels are at their highest immediately after copulation. The hormone is then slowly released from the plug over the next 24 hours as the plug is digested, while MISO transcripts peak at 24 hpm by which time the amount of 20E left in this tissue is negligible. In the case of blood feeding, 20E levels begin to rise within 12 hpbf and reach their highest point 24-30 hpbf (E. G. Kakani and S. N. Mitchell, unpublished), while MISO protein in the ovaries peaks at 36 hpbf, concurrent with a decrease in 20E titers. These data from post-mating and post-blood feeding samples suggest that MISO expression is somehow synchronized with declining ecdysteroid titers, hinting at a possible role of MISO in reducing 20E levels or scavenging the hormone to ensure it does not persist and induce deleterious effects on reproductive tissues. In support of this hypothesis, the co-regulated ETH hormone from the MISO correlation network is released from Inka cells to induce ecdysis only once 20E levels decline. Furthermore, the co-regulated HR4 functions during larval development to terminate ecdysone pulses by repressing the expression of the cytochrome P450 Cyp6t3, a protein that is posited to play a role in the ecdysone biosynthesis pathway, as its silencing reduces ecdysteroid titers in Drosophila. The fact that putatively co-regulated hormones play a role in biological processes occurring at the decline of 20E or directly block further 20E synthesis suggests that perhaps MISO functions to reduce or dampen 20E signaling. Indeed, MISO silencing before mating induced a slower decrease in 20E levels in the atrium following mating, suggesting the hormone persisted in the atrium and was inefficiently scavenged or released from this tissue.

How does MISO affect An. gambiae tolerance to *P. falciparum* infection? Central to finding this mechanism is to determine whether *P. falciparum* infection alters female physiology in ways
that require MISO’s function. Indeed, in a number of experiments comparing the ovaries of females post- \textit{P. falciparum} infection relative to uninfected controls, the abundance of MISO was increased in infected females, though this level of induction varied between replicates (Figure S4). These data suggest increased levels of MISO could be required post-infection to protect egg development. We have also established that the reduced fecundity observed in \textit{P. falciparum}-infected dsMISO females is not due to increased follicular apoptosis, suggesting the absence of MISO may instead lead to impaired follicular growth. Testing of this hypothesis will require staining the ovaries of \textit{P. falciparum}-infected dsMISO females with EdU (5-ethynyl-2’-deoxyuridine, an alternative to BrdU staining that better conserves tissue morphology) to detect possible impairments in growth.

If MISO was important for the regulation of (yet to be confirmed) ovarian hypoxia after blood feeding, it may be possible that \textit{P. falciparum} infections influence this hypoxic response, possibly via the production of ROS. While infection with the rodent malaria \textit{P. berghei} increases H$_2$O$_2$ production in the \textit{An. gambiae} midgut, no similar effect was observed with \textit{P. falciparum} infection, though this parasite appeared to suppress production of ROS \cite{19}. However, other components in the blood meal could be different in \textit{P. falciparum}-infected samples. For example, the blood meal is normally rich in heme molecules that have been shown to interact with oxygen to generate superoxide, which could stimulate more hypoxic signaling (reviewed in \cite{82}). The amount of free heme could be increased in infected red blood cells due to cell lysis during parasite egress, which could in turn induce a greater hypoxic response. Furthermore, other products of heme metabolism, including bilirubin, have been shown to interact with the aryl hydrocarbon receptor (AHR), which in association with HIFβ (ARNT) can induce gene expression of factors involved in metabolism, including cytochrome P450s \cite{83}. Further experiments on whether \textit{P. falciparum} can alter female physiology and/or induce signaling through pathways involved in egg development are required.
Another outstanding question from this study is whether MISO’s role in the atrium or in the ovaries/trachea mediates female tolerance to *P. falciparum*. Our analysis of MISO levels in dsMISO atria and ovaries/trachea relative to controls by Western blotting suggests that while silencing of this gene in atria is highly efficient, it is less effective in the ovaries/trachea, complicating our ability to determine the tissue localization mediating its role in egg development. This difficulty in reducing levels of MISO in the ovaries via RNAi may occur because the high levels of protein produced in one day old females are stored in another tissue or modified within the ovaries, then activated or released upon blood feeding rather than *de novo* synthesized. As a result, dsRNA injection, which occurs in one day old females, may fail to effectively knock-down MISO transcription in the ovaries, as the protein has already been produced. We are currently generating a transgenic MISO knock-out line by CRISPR/Cas9 mutagenesis which may help elucidate the transcription and translation dynamics of this gene in the ovaries. However, the fact that we still see an effect of injection of dsMISO on tolerance to *P. falciparum* despite not detecting a parallel reduction in protein levels in the ovaries suggests the fitness phenotype we observe is at least partially due to MISO synthesized post-mating.

20E is highly conserved as a molting hormone in the insect order *Diptera*. In anautogenous mosquito species, it is also produced post-blood feeding to induce egg development. On the other hand, sexual transfer of male 20E is a derived trait that has evolved only in a subset of *Anopheles* species. Increasing amounts of evidence is emerging from other insects including *Drosophila* and *Aedes* that developmental pathways stimulated by 20E during molting are utilized in the adult stages to stimulate egg development. Remarkably, our data suggest that these pathways may also be conserved to transduce the 20E signal transferred to the female during mating through MISO, and our previous evidence suggests that this protein may have adapted in response to sexually-transferred 20E. We have demonstrated that anopheline species that have evolved this unique mating trait, including the two species of the *Cellia* subgenus *An. gambiae* and *An. stephensi*, do not suffer costs associated with *P. falciparum* infection. However,
the plugless and male 20E-less species *An. albimanus* of the *Nyssorhynchus* subgenus produces fewer eggs when infected with *P. falciparum*. Intriguingly, *Anopheles* species that have this 20E-based mating system are primarily Old World vectors \(^\text{15}\) that have experienced pressure from *P. falciparum* parasites for over 10,000 years \(^\text{84,85}\), but the New World species that lack this mating system have only recently come into contact with *P. falciparum* in the 16\(^{th}\) century due to the transatlantic slave trade \(^\text{86}\). We hypothesize that *P. falciparum* parasites may have adapted to the mosquito vector and learned to utilize conserved 20E signaling for their own development. Furthermore, the parasite may have greatly benefited from the evolution of the 20E-based mating system that drove the female protein MISO to acquire new functions in a subset of *Anopheles*, conferring tolerance to infection.

**MATERIALS AND METHODS**

**Mosquito rearing and mating procedures**

Mosquitoes were reared under standard conditions (26-28˚C, 65-80% relative humidity, 12-hour:12-hour light:darkness photoperiod). For mating and infection experiments conducted in the laboratory with *An. gambiae* (G3 strain), *An. stephensi* (sd500 strain), and *An. albimanus* (STECLA strain), mosquitoes were separated by sex as pupae and raised in cages with 10% glucose supplied *ad libitum*. For *An. gambiae* and *An. stephensi*, matings were performed by adding 4 day-old females to cages containing males at a 1:2 ratio and capturing couples *in copula* \(^\text{13}\) or via the forced mating technique (method available at https://www.beiresources.org/MR4Home.aspx). To capture couples *in copula*, females were introduced 10-20 at a time into cages of males, and when mating couples formed and dropped to the bottom of the cage, modified 50 ml Falcon tubes covered with netting were used to collect the pair. These Falcon tubes were then placed on ice to anesthetize females, and more females were introduced in the cage for maximum one hour to capture couples. Females that failed to mate were screened out from the experiment by checking the abdomens of anesthetized females on a
stereomicroscope Leica M80 with Fluorescence Light Source EL 6000 for autofluorescence resulting from the presence of a mating plug in the female atrium. For the forced mating technique, males were anesthetized on ice, mounted on dissecting pins, and beheaded. The abdomen of these males was then put in contact with the abdomen of anesthetized females, inducing a copulatory response. Females were anesthetized on ice immediately following forced copulation, and the presence of mating plug was assessed via abdominal autofluorescence as described above. For An. albimanus, females were added to cages of males in a 1:2 ratio and allowed to mate for 1 hour, then removed from these cages. Mating status was determined by assessing the presence/absence of sperm in the spermatheca at the time of dissection to assess fecundity and P. falciparum infection. For all species, females were fed at 5 days post-eclosion, and glucose solution was removed from cages 18-24 hours prior to blood-feeding.

For mating and infection experiments with P. falciparum from gametocyte carriers conducted in Burkina Faso, An. coluzzii mosquitoes were used from a colony established in 2008 from wild-caught gravid females collected in the vicinity of Bobo-Dioulasso, Burkina Faso. Mosquitoes were separated by sex as pupae and raised in cages with 5% glucose solution provided ad libitum. Two days post-emergence, females were added to cages of 3-4 day-old males in a 1:2 ratio to permit mating over the following 2 nights. Glucose solution was removed from cages 18-24 hours prior to blood feeding, and females were removed from cages and placed in cups 1-2 hours before blood feeding. Mating status was determined by assessing the presence/absence of sperm in the spermatheca at the time of dissection to assess fecundity and P. falciparum infection.

**RNA interference**

To knock-down gene expression of MISO (AGAP002620) and the control gene GFP, plasmids generated for the publication Baldini et al., PLoS Biology, 2013 were used. A 397 bp region of the coding sequence of MISO and a 495 bp region of GFP were cloned into the pLL10 and pCR2.1 (Thermo Fisher Scientific) plasmids, respectively. For RNAi experiments in the field, the
control gene used was LacZ using a plasmid generated for the publication Blandin et al., Cell, 2004, by cloning a 816 bp region of the bacterial LacZ sequence into the pLL10 plasmid\(^{20}\). Using primers containing the T7 sequence and specific to the plasmid backbone, PCR reactions were performed on 250-1000 pg plasmid DNA to amplify the coding sequence and generate knock-down constructs specific to each gene. The primers used to amplify the MISO and LacZ cassettes were Forward: 5'-TAATACGACTCACTATAGGGCTCGAGGTCGACGGTATCG-3' and Reverse: 5'-TAATACGACTCACTATAGGGCGGCCGCTCTAGAAGTACG-3’ (T7 regions underlined). The primers used to amplify the GFP cassette were Forward: 5'-TAATACGACTCACTATAGGGCCGCGGTGCCTGGAA-3' and Reverse: 5'-TAATACGACTCACTATAGGGGATATCGCAGAATTCGCCC-3'. Double-stranded RNA (dsRNA) was then generated by incubating each PCR product with T7 enzyme 16-20 hours at 37°C using the MEGAscript™ T7 Transcription Kit (Thermo Fisher Scientific), purifying the dsRNA product by phenol-chloroform extraction, and re-suspending purified dsRNA in molecular grade de-ionized water (dH\(_2\)O) at a concentration of 10 µg/µl. 1-day old females anesthetized on Inject+Matic Sleeper TAS pads releasing nitric oxide were injected with 69 nl of 10 µg/µl dsRNA using a Drummond Scientific Nanoject II or III. Injected females were then mated 3 days post-injection and/or blood-fed 4 days post-injection, then used to assess the effect of gene silencing on egg development, \(P.\) falciparum infection intensity and prevalence, gene expression, and protein levels. To assess silencing efficiency in MISO RNAi experiments, 8-12 atria were dissected from dsMISO females 24 hours post-mating and pooled in TRIzol® Reagent. Tissues from dsGFP control females were collected at the same time as experimental groups, and RNA from both pools was extracted in the TRIzol® Reagent. Methods for RNA extraction, cDNA synthesis, and quantitative RT-PCR (qRT-PCR) are outlined in the Gene expression analysis in mosquito tissues by quantitative RT-PCR section. Experiments were discarded if the knock-down efficiency of MISO was lower than 75%, except in field experiments where knock-down efficiency was more variable.
Fecundity assays

In laboratory assays to determine the effect of *P. falciparum*-infected blood feeds on anopheline fecundity, female mosquitoes of the species *An. gambiae*, *An. stephensi*, and *An. albimanus* that were either captured *in copula*, forced mated, or kept virgin at day 4 days post-eclosion (as outlined in Mosquito rearing and mating procedures section above) were blood fed 5 days post-eclosion (1 day post-mating) on blood meals either infected with *P. falciparum* from the NF54 strain, or on a sample containing blood from the same NF54 culture but with heat-inactivated gametocytes. The protocol for heat-inactivating *P. falciparum* cultures is described below in the Experimental infections methods section. 48 hours post-blood feeding, females were collected into 80% ethanol in the infection glove box using a Bioquip ethanol aspirator, kept at -20°C for a minimum of 5 minutes in 80% ethanol, then dissected at a Nikon SMZ1000 stereomicroscope to collect ovaries and count the number of eggs developed for each female.

In laboratory assays to determine the effect of *P. falciparum*-infected bloodfeeds on the fecundity of *An. gambiae* females silenced for MISO, females injected with dsRNA targeting MISO or the control GFP gene were mated and fed 24 hours post-mating on either uninfected or NF54-infected blood. 48 hours post-blood feeding, females were collected from the infection box and dissected to count the number of eggs developed for each female as described above.

For experiments conducted in Burkina Faso to assess the impact of infections of *P. falciparum* from gametocyte carriers on fecundity in females depleted of MISO, females injected with dsRNA targeting MISO or the control LacZ gene were allowed to mate over 2 nights and fed the morning after the second night on blood collected from gametocyte donors. 8 days post-infection, females were collected from cages, anesthetized on ice, and dissected to count the number of eggs developed, the presence of sperm in the spermatheca, and the number of oocysts in the midgut.
Experimental infections with NF54 *P. falciparum*

All cultures were maintained at 37°C in an incubator gassed to 5% oxygen, 5% carbon dioxide, 90% nitrogen. Asexual cultures of NF54 were maintained below 2% parasitemia at 5% hematocrit (O+ red blood cells, RBC) in complete medium (RPMI 1640 with L-glutamine and 25 mM HEPES, 10 µg/ml hypoxanthine, 0.2% sodium bicarbonate, 10% O+ human serum). To induce gametocytogenesis, cultures were grown without splitting to parasitemias of 3-10%, then split to 2% parasitemia in complete media at 5% hematocrit (using less than 1-week old RBC) to a total volume of 15 ml. 5 ml of this culture was aliquoted to 3 separate wells of a 6 well plate and cultured for 14-20 days, changing the media every day. To determine the exflagellation rate of a gametocyte culture to assess its suitability for an infectious feed, an equal amount of culture was taken up from each of the three induction wells to a final volume of 400 µl and spun down at 1500 rpm for 1 minute at room temperature. 11.5 µl of the infected RBC pellet was added to 10 µl of human serum heated to 37°C, resuspended, and incubated on a slide with a coverslip at room temperature for 15-20 minutes. The number of exflagellations per field was determined by microscopy at 100X for five fields to determine approximate infectivity of a culture. Gametocyte cultures with exflagellation rates greater than 10 were used for mosquito infections.

For the infectious feed, gametocyte cultures were spun down at 37°C at 1800 rpm for 2 minutes, then diluted 1:10 in a 1:2 mix of fresh RBC and human serum at 37°C. To heat-inactivate gametocytes as an uninfected control, an aliquot of this resuspended culture was incubated at 42°C shaking at 500 rpm for 15 minutes \(^{30}\). For completely uninfected blood controls, fresh RBC was added to human serum heated to 37°C in a 2:5 ratio. For infected, heat-inactivated, and uninfected experimental feeds, 160 µl of these samples was loaded into custom-designed Chemglass Life Sciences glass membrane feeders sealed with parafilm and heated to 37°C with a Haake D1 Immersion Circulator, allowing the feed of experimental and control groups simultaneously on the same gametocyte culture. Mosquitoes were allowed to feed for 30-60
minutes, adding more culture as necessary, and all non- and partially-fed females were removed from cages following the infection using a Bioquip ethanol aspirator.

**Experimental infections with P. falciparum from gametocyte carriers**

*Ethics statement:* Procedures involving human subjects were conducted in accordance with protocols approved by the Institutional Ethics Committee of the Centre Muraz, Bobo-Dioulasso, Burkina Faso and the Ethics Committee of the General Administration Department of the Ministry of Health of Burkina Faso.

Surveys for *P. falciparum* gametocyte carriers were conducted in the villages surrounding Bobo-Dioulasso, Burkina Faso by collaborators at the IRSS/Centre Muraz. Blood smears were collected from pinprick samples from children ages 5-13, stained with 10% Giemsa, and assessed for *P. falciparum* infection levels by microscopy. The number of asexual and sexual parasites present per 1000 white blood cells was counted, and translated to gametocytes per µl of blood using the conversion 8000 white blood cells/µl of blood. Volunteers with gametocytemias of 70-320 gametocytes/µl of blood were enrolled for the study following parental consent. For infectious feeds, 2 ml of blood was drawn from gametocyte carriers, spun down at 37˚C at 1800 rpm for 5 minutes, and serum was removed and replaced with an equal volume of *Plasmodium*-naïve serum. The sample was resuspended and loaded into custom-designed glass membrane feeders sealed with Parafilm and heated to 37˚C with a Haake D1 Immersion Circulator, allowing the feed of experimental and control groups simultaneously on the same gametocyte carrier sample. Mosquitoes were allowed to feed for 60-90 minutes, adding more culture as necessary, and all non- and partially-fed females were removed from cages following the infection.

**Assessing P. falciparum infection prevalence and intensity in the mosquito midgut**

7-8 days post-infection, the midguts of mosquitoes were dissected and stained in 2 mg/ml mercury dibromofluorescein disodium salt (mercurochrome) for 12-15 minutes, then loaded onto 10-well
slides in 0.2 mg/ml mercurochrome and covered with a coverslip for visualization and counting by microscopy.

**Polyclonal MISO antibody**
A polyclonal antibody against MISO was raised in rabbit using the peptide antigen CSNGPSSSYGPRNT and affinity-purified by a commercial supplier (GenScript USA Inc)⁵.

**Co-immunoprecipitation assays**
The Dynabeads™ Co-immunoprecipitation Kit (Thermo Fisher Scientific) was utilized, and the kit instructions for detection by Western Blot or Silver Stain were followed with some modifications. MISO polyclonal antibody was conjugated to Dynabeads™ M-270 Epoxy at a ratio of 20 µg antibody per 1 mg beads overnight at 37°C 16-24 hours on a roller. Following this incubation, the antibody-conjugated beads were washed and stored at a concentration of 10 mg/ml at 4°C. Three different tissue samples were collected for co-immunoprecipitation (co-IP) in extraction buffer [1X IP Buffer from kit, 150 mM NaCl, 1X cOmplete™, EDTA-free protease inhibitor cocktail (Sigma-Aldrich)]: 150 mated atria, 450 blood-fed ovaries 36 hpbf, and 450 ovaries from 1 day-old females. Samples were stored at -80°C until extraction, at which point tissues were homogenized with a hand-held homogenizer and pestles, incubated on ice for 30 minutes, then spun down at 14,000 rpm at 4°C for 10 minutes to remove cell debris. Samples were quantified by BCA assay (Pierce®, Thermo Fisher Scientific). Cell lysates were incubated with 1.5 mg of antibody-coupled beads either 1 hour or overnight at 4°C on a roller. Bead complexes were then washed, with each wash collected, then incubated 15 minutes in EB buffer on a roller at room temperature. This supernatant containing protein complexes, as well as 2-5% of the pre-IP sample, 2-5% of the post-IP sample, and 2-5% of each of the washes was run by Western blot (see Western blotting section below) and by Silver Stain to detect the presence of MISO and any co-IP proteins.
Gels containing protein complexes and all other control samples for co-IP experiments were stained with the Pierce® Silver Stain Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. In brief, gels were fixed in 30% ethanol:10% acetic acid solution, washed in 10% ethanol solution, incubated with Sensitizer Working Solution (1 minute) and Stain Working Solution (30 minutes), then developed in Developer Working Solution for 2-3 minutes until bands appeared at the desired intensity. The reaction was stopped with a 5% acetic acid solution, and gels were visualized and imaged with an Alphalmager® EC (Alpha Innotech).

Correlation network analysis of transcriptional datasets

Raw microarray files were retrieved from ArrayExpress (V.A. Ingham et al. 2014 38, P. Gabrieli et al. 2014 6), VectorBase (Baker et al. 2011 39, Marinotti et al. 2006 36, Vannini et al. 2014 37) or in the case of unpublished data, directly from Catteruccia et al. (Table S1). Arrays retrieved from VectorBase were analyzed using the in-built Affymetrix platform GEO2R 87,88, others were analyzed using the limma R package 89, as described in 38. The fold change and Q values generated in data outputs from limma anaylsis and GEO2R were combined. The resultant large dataset was used in an app developed in the R package Shiny 34,35 by Ingham et al. The aim of this app is to interactively explore the expression of a transcript of interest, in this case MISO, and to identify putative functions/pathways by identifying strongly correlated transcripts. To identify these strongly correlated transcripts, Pearson’s pairwise correlation matrices were produced using the stat package in R 35, with a cut-off of \( \rho > \pm 0.7 \). Cytoscape 3.6.0 90 was used to visualize the correlation network. An edge-weighted directed layout was used with edge weighting based on correlation value. The degree of connectivity was also illustrated by using \( \rho \) to define edge width.
Gene expression analysis in mosquito tissues by quantitative RT-PCR

Mosquito tissues were dissected in 1X phosphate-buffered saline (1X PBS) and collected in TRIzol® Reagent on ice at different timepoints post-mating and post-blood feeding to assess expression of genes of interest. Three replicates of each of the following experiments were collected. For the life-time transcriptional profiling of G3 females post-eclosion, post-mating, and post-blood feeding, 10-15 ovaries were collected at 1 and 4 days post-eclosion in 100 µl TRIzol® Reagent. On day 4 post-eclosion, females were separated into two groups, and one group was mated while the other was kept virgin. 24 hpm, on day 5 post-eclosion, an ovary tissue pool was collected from the virgin and mated groups (0 hpbf), then a subset of females from both groups were blood fed while the rest were kept non-blood-fed, and ovary pools of each of these 4 groups were collected at 3, 24, and 48 hpbf in three biological replicates. For the post-blood feeding transcriptional timecourse, females were injected with dsMISO or dsGFP, mated 3 dpi, and blood fed 24 hpm. The ovaries of 10-12 females were collected immediately prior to blood feeding (0 hpbf), as well as at 3, 24, and 48 hpbf in three biological replicates. For the post-mating transcriptional timecourse, females were injected with dsMISO or dsGFP, mated 3 dpi, and 8-12 atria and ovaries were dissected as a single tissue and pooled in 100 µl TRIzol® Reagent at 6, 24, and 48 hpm in three biological replicates. Samples were stored at -80°C until RNA was extracted, at which point they were thawed on ice and homogenized with a hand-held homogenizer and pestles. RNA was extracted in TRIzol® Reagent using 1-bromo-3-chloropropane and precipitated in isopropanol. RNA was quantified using a Nanodrop 2000c, and 2 µg total RNA from ovary and atria+ovary tissue pools were treated with 2 units TURBO DNase (Thermo Fisher Scientific). For atrial pools where the total amount of RNA was generally under 2 µg, all samples within the replicate including controls were scaled to the sample with the lowest amount of RNA, then treated with 2 units TURBO DNase. The DNase-treated RNA samples were then loaded into 100 µl cDNA synthesis reactions in mixtures with reagents at the following final concentrations: 1X First Strand Buffer, 5 mM DTT, 500 µM dNTP mix, 0.4 units RNaseOUT™
recombinant ribonuclease inhibitor, 2.5 µM random hexamers, and 1.25 units M-MLV reverse transcriptase (all reagents from Thermo Fisher Scientific).

Primer sets suitable for qRT-PCR were designed using Primer-BLAST (NCBI) and when possible were designed to span exon-exon junctions (see Table S3 for list of primers used for qRT-PCR). Individual primer sets were tested on representative cDNA pools for optimal final primer concentrations of 300 nM or 900 nM, as well as on serial dilutions of representative cDNA pools. Quantification was done using SYBR Green Detection, mixing 7.5 µl 1X Fast SYBR™ Green Master Mix (Thermo Fisher Scientific), 5 µl of a 1:10 dilution of cDNA, and primers at optimal concentrations, in duplicate for each sample on a StepOnePlus Real-Time PCR System (Applied Biosystems). Relative quantification of gene expression was calculated using the ∆Cₜ method, with the ribosomal protein RpL19 (AGAP004422) used as the housekeeping gene. Samples were excluded from the analysis if the presence of 2 melt curves was detected or if amplification occurred in the non-template controls. For the post-blood feeding transcriptional timecourse of dsMISO and dsGFP females, the relative expression of HIF genes in samples at each of the blood-fed timepoints (3, 24, and 48 hours) were normalized to the relative expression of the non-blood-fed controls of that injection treatment using the ∆∆Cₜ (Livak) method.

**Western blotting**

8-12 females were dissected to collect atria and ovaries into separate pools at different timepoints post-mating and post-blood feeding. Tissues were collected on ice and stored at -80°C in protein extraction buffer (PEB) containing the following reagents at the following final concentrations: 25 mM Tris HCl pH 7.4, 150 mM sodium chloride, 0.1% sodium dodecyl sulfate (SDS), 1% Triton-X100, 10 mM EDTA pH 8, 1X cOmplete™, EDTA-free protease inhibitor cocktail (Sigma-Aldrich), and 1% phosphatase inhibitor (Sigma-Aldrich). Samples were thawed on ice and homogenized with a hand-held homogenizer and pestles, then spun down at 14,000 rpm at 4°C for 10 minutes. The supernatant of each sample was collected and quantified in a microplate by
Bradford assay (Bio-Rad). Approximately 25 µg of each sample was denatured and reduced via a 10-minute incubation at 70°C in 1X NuPAGE LDS Sample Buffer (Thermo Fisher Scientific) and 100 mM DTT. Samples were then run on pre-cast 4-12% Bis-Tris NuPAGE gels (Thermo Fisher Scientific), transferred to PVDF membranes using the iBlot 2 Transfer System (Thermo Fisher Scientific), blocked, then incubated shaking overnight at 4°C with the affinity-purified polyclonal rabbit MISO antibody at a concentration of 1.5 µg/ml in blocking buffer. Following washes with 1X PBS-T (1X PBS + 0.1% Tween-20), membranes were incubated with anti-rabbit IRDye800 (LI-COR) diluted 1:15,000 in blocking buffer + 0.01% SDS 1 hour at room temperature, washed, then incubated with an anti-actin rat monoclonal antibody (Abcam) at a concentration of 0.21 µg/ml in blocking buffer 1 hour at room temperature. Following washes, the membrane was probed with anti-rat IRDye680 (LI-COR) diluted 1:20,000 in blocking buffer + 0.01% SDS for 45 minutes at room temperature, washed, then imaged using a LI-COR Odyssey Imaging System.

20E injection

Three-day-old adult female mosquitoes of the G3 colony were injected with 138 nl of a 38 mM 20E solution diluted in 10% ethanol (equivalent to 2.5 µg 20E per mosquito) using a Drummond Scientific Nanoject II. Control females were injected with 138 nl of a 10% ethanol solution as a negative control, and uninjected females were bloodfed as a positive control. 24 hpi, females were dissected in 1X PBS to collect 10-12 ovary pairs per tissue pool in 10 µl PEB (see Western blotting section above). 10-12 positive control ovary pairs were collected in 20µl PEB 36 hpbf. Samples were analyzed by Western blot assay probing for MISO as described in the Western blotting Methods section.

Inactivation of 20E post-blood feeding by E22O oxidase injection

Three-day-old adult female mosquitoes of the G3 colony were injected with 207 nl of 0.5 mg/ml E22O oxidase using a Drummond Scientific Nanoject II or III. Control females were injected with
equivalent volumes of 0.5 mg/ml BSA. 8-10 hpi, females were blood fed, and 24 and 36 hpbf females were dissected in 1X PBS to collect 10-12 ovary pairs per tissue pool in 20 µl PEB (see Western blotting section above). Samples were analyzed by Western blot assay probing for MISO as described in the Western blotting Methods section.

Immunofluorescence assays

Ovaries from G3 females were dissected in 1X PBS prior to blood feeding (0 hpbf) and at 6 and 24 hpbf, and fixed in immunostain baskets in 4% paraformaldehyde (Electron Microscopy Sciences) solution diluted in 1X PBS for 30-40 minutes at room temperature. Tissues were washed in 1X PBS, then blocked and permeabilized in blocking solution (1% bovine serum albumin (BSA) + 0.1% Tween-20 in 1X PBS) for 30-60 minutes. Tissues were then incubated with anti-MISO antibody at a concentration of 3 µg/ml in blocking solution overnight at 4˚C. As negative controls for this experiment, a peptide competition assay (PCA) was performed. For each reaction, 3 µg/ml MISO antibody was incubated with a 200-fold molar excess of the immunizing peptide in blocking solution on a roller for 20 hours at 4˚C. The following day, PCA samples were spun down at maximum speed (4000 rpm) for 15 minutes at 4˚C, and the supernatant was used to stain non-blood-fed and blood-fed ovaries overnight at 4˚C. The MISO antibody used to positively stain tissues was treated the same as the PCA samples, but without immunizing peptide added. Following overnight incubation, all samples were washed in blocking solution then incubated in a 1:1000 dilution of anti-rabbit Alexa546 antibody in blocking solution for 1 hour at room temperature. Samples were then washed in blocking solution and incubated with 1 µg/ml DAPI in blocking solution at room temperature for 10 minutes. Samples were washed then mounted into Vectashield mounting medium (Vector Laboratories) on slides, sealed under coverslips, and stored in the dark at 4˚C until visualized and imaged on a Leica SPE Confocal Microscope. Images were analyzed on Leica Application Suite X, and any intensity adjustments of images in the DAPI or MISO-Alexa546 channel at 0 and 24 hpbf were done uniformly on both
MISO-incubated and PCA-incubated samples. At 6 hpbf, the intensity in the MISO-Alexa546 channel was increased relative to 0 and 24 hpbf images in order to better visualize oocyte staining, though this increased intensity adjustment was kept consistent between MISO-incubated and PCA-incubated samples.

**Promoter analysis with Genomatix MatInspector software**

Genomatix has developed the MatInspector software tool to find DNA sequences in the promoter region of a gene that match putative transcription factor (TF) binding sites compiled into a large library of matrix families. These matrix families are composed of similar and/or functionally related TF binding sites, and for this analysis, matrices belonging to the subgroups Insects, Vertebrates, and General Core Promoter Elements were selected from the Matrix Library 11.0. Core similarity (matching to the most highly conserved 4 core nucleotides in a TF binding site) cut-off was set to 0.75 (a minimum of 3 of 4 highly conserved nucleotides must be present). Matrix similarity (calculated by matching nucleotides in the promoter region to highly and less conserved regions) was set to an optimized value described in $^{61}$. The analysis was conducted on the putative promoter of MISO as identified in the ElDorado 12-2017 library, predicted in the Anopheles genome using Genomatix PromoterInspector algorithm $^{91}$ (genomic site 24,260,709-24,261,809, 1101 bp). This analysis returned 408 putative TF binding sites in the MISO promoter region, 7 of which were sites for HIF or aryl hydrocarbon receptor (AHR)/ARNT factors as identified in the Vertebrate subgroup (V) ($\textbf{Table S2}$). All Matrix similarity values for these TF binding sites were greater than the calculated Optimized value and were above the MatInspector cut-off for good matches at 0.8.

**In situ detection of apoptosis**

Ovaries from mated dsMISO and dsGFP females were dissected in 1X PBS 18 hpbf on a $P. falciparum$-infected bloodmeal, fixed, and washed as described in the **Immunofluorescence**
assays Methods section. The tissues were then transferred out of immunostain baskets onto welled glass slides in a humidity chamber, and apoptosis was detected using the ApopTag® Fluorescein In situ Detection Kit (Millipore). Like a TUNEL assay where TdT enzyme catalyzes the addition of nucleotide triphosphates to free 3’ hydroxyl groups that are present at higher concentrations in apoptotic cells due to DNA strand breaks, the TdT Enzyme Mix in the ApopTag® kit contains TdT enzyme and nucleotides modified with digoxigenin (DIG-dNTPs). DNA fragmentation in tissues is therefore assessed by incubating the tissues in the TdT enzyme mix, then detecting the level of DIG-dNTP incorporation using an anti-DIG antibody. The methods used in these experiments were a modified version of the kit’s protocol for “Fluorescent staining of paraffin-embedded tissues.” Ovaries were incubated in Equilibration Buffer, followed by incubation in TdT Enzyme Mix (TdT enzyme and Reaction Buffer containing DIG-dNTPs mixed at a 3:10 ratio) at 37°C. Following a 2.5-hour incubation in this enzyme mix, the reaction was stopped in the ovaries using Stop/Wash Buffer, and tissues were washed with 1X PBS + 0.1% Triton-X100. The samples were incubated with anti-DIG antibody for 1 hour at room temperature, washed, mounted in Vectashield + DAPI (Vector Laboratories), sealed under a coverslip, and stored in the dark at 4°C until visualized and imaged on an Axio Observer Z1 inverted microscope with an Apotome 2 slider module (Zeiss).

Caspase activity in the ovaries of P. falciparum-infected females
Caspase-3 and 7 cleave proteins at the specific amino acid sequence Asp-Glu-Val-Asp (Aspartic acid-Glutamic acid-Valine-Aspartic acid, also referred to as DEVD), and activation of these enzymes has been shown to be important for inducing apoptosis. The EnzChek® Caspase-3 Assay Kit #2 (Molecular Probes) utilizes a bisamide derivative of rhodamine 110, rhodamine 110 bis-N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide (or Z-DEVD-R110), as a substrate to detect caspase-3/7 activity. Upon caspase cleavage of DEVD, the non-fluorescent Z-DEVD-R110 molecules are converted to the fluorescent R110 molecule in two steps, allowing
the level of enzymatic activity to be monitored and recorded at fluorescence excitation/emission of 496/520 nm. To measure the level of caspase-3/7 activity in the ovaries of dsMISO and dsGFP females fed a *P. falciparum*-infected bloodmeal, 6 pairs of ovaries were collected in duplicate pools for each injection treatment at 3, 12, and 24 hpf in the kit’s 1X Cell Lysis Buffer supplemented with 1X cOmplete™, EDTA-free protease inhibitor cocktail (Sigma-Aldrich) and stored at -80°C. Samples were thawed on ice, homogenized with a hand-held homogenizer and pestles, resuspended in a final volume of 50 µl, incubated on ice for 30 minutes, and spun down at 14,000 rpm at 4°C for 10 minutes. The supernatant of each sample was collected and loaded into a microplate, and 50 µl 2X substrate working solution (containing reaction buffer and Z-DEVD-R110) was added to each well, with a final Z-DEVD-R110 concentration of 25 µM per well. An R110 standard curve was also prepared and loaded on the microplate. The plate was covered and incubated at room temperature, and fluorescence was measured on a spectrophotometer (excitation/emission 496/520 nm) after 30, 60, 120, and 180 minutes of incubation with the Z-DEVD-R110 substrate.

**Statistical analysis**

All statistical analysis was performed using GraphPad Prism® Version 7.0 and JMP® Pro Version 13.0. The statistical test performed for each experiment is detailed in the text.

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REFERENCES


CHAPTER 3:

Different impact of Wolbachia on Plasmodium development in natural and laboratory Anopheles infections

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Author Contributions: P. M., W.R.S., and F.C. designed the experiments. P.M. and W.R.S. performed the experiments, with only W.R.S. performing the fluorescent in situ hybridization experiments. P.M. and W.R.S. established the wAnga-infected mosquito lab colony from natural An. coluzzii mosquitoes. F.B. collected and analyzed Wolbachia presence in samples from 2011 and 2013. P.M., W.R.S., and F.C. analyzed the data. L.M.C. and C.O.B. mathematically modeled
the data. S.S., R.K.D., and A.D. provided samples for the analysis. P.M., W.R.S., L.M.C., and F.C. wrote the manuscript. This chapter comprises work published in:


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ABSTRACT

The maternally inherited α-proteobacterium Wolbachia has long been proposed as a tool to block transmission of devastating mosquito-borne infectious diseases like dengue and malaria. Here we study the reproductive manipulations induced by a recently identified Wolbachia strain that stably infects natural mosquito populations of a major malaria vector, Anopheles coluzzii, in Burkina Faso. We determine that these infections significantly accelerate egg-laying but do not induce cytoplasmic incompatibility or sex-ratio distortion, two parasitic reproductive phenotypes that facilitate the spread of these bacteria within insect hosts. Analysis of 221 blood-fed females collected from houses shows a negative correlation between the presence of Plasmodium parasites and Wolbachia infection. A mathematical model incorporating these results predicts infection with these endosymbionts would substantially reduce malaria prevalence in human populations. However, experimental infections of a laboratory-adapted Wolbachia-infected An. coluzzii mosquito line with Plasmodium falciparum NF54 show no effect of Wolbachia on Plasmodium infection intensities or prevalence. These data suggest that Wolbachia may be an important player in malaria transmission dynamics in sub-Saharan Africa, but that ecological factors or Wolbachia-Plasmodium interactions specifically occurring in natural populations are key to this effect.

INTRODUCTION

Every year nearly 200 million people contract malaria and around 450,000 people die from the disease, mostly young children in sub-Saharan Africa. Malaria transmission depends on the complex ecological determinants that drive population dynamics of Anopheles mosquitoes, the vectors of human malaria parasites. Measures aimed at the mosquito vector have been the mainstay of malaria control strategies and have achieved significant decreases in the global burden of this disease over the last decade, primarily attributable to the widespread use of long-
lasting insecticide-treated nets (LLINs) \(^3\). However, insecticide-based prevention tools are severely threatened by the rapid spread and global distribution of insecticide resistance in anopheline populations \(^4,5\), making the development of new insecticide-free tools for reducing malaria transmission a crucial priority \(^6\).

The use of *Wolbachia* bacteria – intracellular endosymbionts of arthropods and nematodes – has long been suggested for the control of mosquito populations transmitting viral or parasitic pathogens such as dengue fever and malaria given their profound effects on both insect physiology and pathogen development \(^7\). These bacteria are best known as active modulators of host reproduction via the induction of mechanisms that promote their rapid invasion of insect host populations. The most commonly observed of these phenotypes is cytoplasmic incompatibility (CI), which prevents uninfected females from producing viable progeny after mating with infected males due to chromosomal segregation defects in the early cellular divisions of the fertilized egg (reviewed in \(^8\)). However, infected females are able to produce viable progeny regardless of whether they mate with infected or uninfected males through still unknown rescue mechanisms, an effect that favors *Wolbachia*’s spread \(^9,10\). Factors involved in CI were recently described and are in fact proteins encoded by the *Wolbachia* prophage WO \(^11,12\). Additional phenotypes include vertical transmission from mother to progeny and a female bias in the sex ratio through selective male killing, male feminization, and female parthenogenesis (where unfertilized eggs develop as females) \(^13-15\). Combined with these properties, *Wolbachia* infections in many mosquito species can prevent pathogen development and block disease transmission from vector to human. The list of vector-borne pathogens affected by *Wolbachia* is broad and includes viral pathogens causing dengue, yellow fever, West Nile, and Chikungunya, as well as *Plasmodium* parasites \(^16-20\), suggesting a general mechanism of pathogen blocking by an upregulated immune response in the mosquito host \(^21-23\), although not in all cases \(^18,24\).
We have recently identified stable *Wolbachia* infections in natural populations of two important malaria vectors, *Anopheles gambiae* and *An. coluzzii*, in Burkina Faso, a country with a high malaria burden. Due to several negative reports, it was previously believed that these bacteria were unable to infect *Anopheles* species, as also suggested by the difficulties to generate stably transinfected *Anopheles* lines in the laboratory. Consequently, blocking of *Plasmodium* parasites was initially demonstrated using either transient injections of non-native *Wolbachia* strains into adult *Anopheles* females, or a bird model of malaria infection transmitted by *Aedes* mosquitoes. Our study showed that *Wolbachia* were transmitted from females to progeny with an average transmission frequency of 68% (ranging from 56-100%) and determined that the strain identified in *Anopheles* (which we named *wAnga*) belongs to a new arthropod-specific supergroup. *Wolbachia* infections have since been confirmed in a subsequent study of *An. coluzzii* mosquitoes from the same region of Burkina Faso, as well as in a survey of *An. gambiae* and *An. coluzzii* mosquitoes in Mali.

As *Wolbachia* can profoundly perturb insect ecology, behavior, and physiology and can significantly reduce transmission of some human pathogens, our discovery provides an unprecedented opportunity to assess the physiological and reproductive impacts of *wAnga* infection on anophelines and to determine how these effects influence the dynamics of malaria transmission. Here we elucidate the complex relationships between *Anopheles* mosquitoes, *Plasmodium* parasites, and *Wolbachia* endosymbionts in natural populations. We find that *wAnga* infections are persistent in *An. coluzzii* over several years, proving these bacteria are stable residents in these mosquito populations, and are also detected in another important malaria vector, *Anopheles arabiensis*. While crosses between infected and uninfected individuals do not show evidence of CI, infected females lay eggs more rapidly than uninfected individuals. Sampling of natural populations shows that *Wolbachia*-positive females are infected with *Plasmodium* parasites at significantly lower frequencies than *Wolbachia*-negative individuals, unveiling a
negative correlation between these endosymbionts and deadly malaria parasites. Modeling the effects of these endosymbionts on malaria transmission dynamics reveals that natural *Wolbachia* infections may have a significant impact on the prevalence of malaria in human populations in sub-Saharan Africa. However, hurdles remain in determining the mechanism underlying the negative correlation between *Wolbachia* and *Plasmodium* infection, as laboratory infections of a lab-adapted wAnga-infected *An. coluzzii* line with *P. falciparum* NF54 did not demonstrate pathogen-reducing effects of *Wolbachia*. These results suggest ecological factors important to natural *Wolbachia*-*Plasmodium* interactions may be absent in laboratory infections.

**RESULTS**

*Wolbachia* infections are stable and localize in the oocytes

We returned to Vallée du Kou near Bobo-Dioulasso, Burkina Faso, in 2014 to determine the prevalence of *Wolbachia* infections in *An. coluzzii* populations. We collected 602 mosquitoes and identified infections at a frequency of 46% (275/602) (Table 3.1). Furthermore, analysis of samples collected in 2013 in the town of Soumousso found wAnga at a frequency of 33% (16/49) in *An. arabiensis* (Table 3.1), a species not effectively targeted by LLINs and indoor residual sprays (IRS) as it primarily blood feeds and rests outdoors. The prevalence of wAnga was moderate compared to that observed in naturally infected Aedes and Culex mosquitoes, although this may be due to difficulties in detection caused by low intensity of infection in some samples. When considering all data since 2011, prevalence was variable between years (between 19–46%) (Table 3.1), suggesting physiological or ecological factors may influence *Wolbachia* dynamics over time.
Table 3.1 | wAnga prevalence across several years in Burkina Faso.

<table>
<thead>
<tr>
<th>Year</th>
<th>Anopheles arabiensis (Soumousso)</th>
<th>Anopheles coluzzii (VK5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>n.d.</td>
<td>7/34 (19%)*</td>
</tr>
<tr>
<td>2013</td>
<td>16/49 (33%)</td>
<td>19/91 (21%)*</td>
</tr>
<tr>
<td>2014</td>
<td>n.d.</td>
<td>275/602 (46%)</td>
</tr>
</tbody>
</table>

Mosquito samples collected in two villages (Soumousso and VK5) near Bobo-Dioulasso are shown. Samples from 2011 represent males and females captured in mating swarms, while 2013 samples are blood-fed females collected from the walls of houses. Samples from 2014 represent blood-fed females from houses and also male and female adults raised from field-collected larvae and eggs. * indicates data previously published in Baldini et al. 2014.

With the aim to facilitate detection of these bacteria within the female, we established a stable wAnga-infected mosquito line in our laboratory after colonization of An. coluzzii populations from the Vallée du Kou villages. We dissected ovaries from females and visualized wAnga at a similar frequency (43%) within the ovarian follicles by fluorescent *in situ* hybridization (FISH) experiments using a DNA probe that specifically hybridizes to *Wolbachia* 16S nucleic acid sequences (Figure 3.1a). In control experiments, no signal was detected in ovaries dissected from females treated with tetracycline to clear wAnga infection (Figure 3.1b), nor when using an excess of unlabeled probe of identical sequence in competition with the labeled probe (Figure 3.1c).
Figure 3.1 | *wAnga localizes to the female germline.* *wAnga* (in green, central panels) was visualized in the ovaries of 14-16 day-old *An. coluzzii* females by FISH. (a) *wAnga* is detected in ovarian follicles using a Cy3-labeled probe specific for 16S DNA (white arrows). (b) *wAnga* is absent from the follicles of tetracycline-treated control females and (c) in follicles of infected females in which the labeled probe was in competition with an identical unlabeled probe (1:20 labeled:unlabeled). DNA is labeled with DAPI (in blue, left panels). Scale bar = 20 µm.

**wAnga does not induce CI but affects oviposition**

Many *Wolbachia* strains induce CI when an infected male mates with an uninfected female, producing mostly inviable progeny. To determine whether natural *Wolbachia* infections induce CI and/or other reproductive phenotypes in field *An. coluzzii*, we collected eggs from blood-fed females in houses or larvae from breeding sites and, after adult emergence, performed crosses between virgin males and females, using a force-mating technique, as field mosquitoes of this species do not mate in captivity. After mating, females were blood fed and allowed to lay
eggs individually in separate oviposition containers. We measured the number of eggs developed to assess the impact of infection on fecundity, and the larval hatch rates to measure effects on fertility and therefore detect a possible occurrence of CI. We also scored the sex ratio of the progeny of each individual female by letting larvae grow to adulthood and counting males and females to determine whether \( w \text{An} \)ga induces sex distortions in these mosquitoes. Both parents of each brood were genotyped and screened for \( W \text{olbachi}a \) post hoc, and crosses between \( W \text{olbachi}a \)-positive males and \( W \text{olbachi}a \)-negative females (i.e. those potentially inducing CI, shown in blue in Figure 3.2) were compared to all other cross combinations (\( W \text{olbachi}a \)-positive males and females; \( W \text{olbachi}a \)-negative males and females; \( W \text{olbachi}a \)-negative males and \( W \text{olbachi}a \)-positive females). Although egg inviability was slightly higher in the crosses in which CI is predicted to occur, we did not observe any significant effect of \( w \text{An} \)ga on egg inviability between our crosses, which suggests that this \( W \text{olbachi}a \) strain does not cause detectable CI in natural \( A. \text{coluzzii} \) populations (Kruskal-Wallis test, \( \chi^2 = 1.25 \), d.f. = 3, \( p > 0.05 \)) (Figure 3.2a). Moreover, we found no difference in the number of eggs developed (Kruskal-Wallis, \( \chi^2 = 4.09 \), d.f. = 3, \( p > 0.05 \)) (Figure 3.2b), eggs laid (Kruskal-Wallis, \( \chi^2 = 0.686 \), d.f. = 3, \( p > 0.05 \)) (Figure S6) or in the progeny sex ratio (Student’s t-test on log\(_{10}\)-transformed ratios, \( t = 0.257 \), d.f. = 26, \( p > 0.05 \)) (Figure 3.2c).
Figure 3.2 | Analysis of reproductive phenotypes induced by wAnga. (a-c) An. coluzzii females and males collected as eggs or larvae from Vallée du Kou were bred to adulthood and forced mated, and after blood feeding the reproductive output of females was individually scored. wAnga-infected (green) and uninfected (grey) females (right) and males (left) were identified by 16S nested PCR post hoc, and mating couples were divided into 4 groups (wAnga-negative couples, dark grey; wAnga-positive couples, purple; wAnga-positive females mated to wAnga-negative males, red; wAnga-negative females mated to wAnga-positive males, blue). (a) Egg inviability was calculated by dividing the number of infertile and unhatched eggs in each brood by the total number of eggs 4 days after egg-laying. CI, which would manifest as higher inviability in the cross between wAnga-negative females and wAnga-positive males (in blue), was not observed (Kruskal-Wallis test, $\chi^2 = 1.25$, d.f. = 3, $p > 0.05$). (b) The total number of eggs produced by each female was determined by adding the number of eggs laid to the number of eggs remaining in the ovaries at the time of dissection (Kruskal-Wallis, $\chi^2 = 4.09$, d.f. = 3, $p > 0.05$). (c) Hatched larvae from each brood in (a) were raised to adulthood and sex was scored. The sex ratio of each brood was calculated as the number of female progeny divided by the total progeny. (Student’s t-test on log$_{10}$-transformed ratios, $t = 0.257$, d.f. = 26, $p > 0.05$). (d) Blood-fed females were collected from the walls of houses in Vallée du Kou and two days after collection placed in individual oviposition containers to record the timing of egg-laying. The cumulative proportion of females that laid on each of 3 consecutive nights following access to an oviposition site is plotted. wAnga-infected females (green line) laid eggs more quickly than uninfected females (grey line) (log-rank test, $\chi^2 = 32.36$, d.f. = 1, $p < 0.0001$).
We next assessed whether egg-laying behavior is altered by wAnga infection. To this end, blood-fed females from houses in the Vallée du Kou were placed in individual cups two days post-blood feeding, and oviposition rates were counted daily. After assessing their infection status post hoc, we determined that wAnga-infected females had laid eggs significantly more quickly than uninfected females (on average $0.73 \pm 0.11$ days, log-rank test, $\chi^2 = 32.36$, d.f. = 1, $p < 0.0001$) (Figure 3.2d). This shortened oviposition timing could increase the number of gonotrophic cycles over the course of a female’s lifespan, and suggests that wAnga-infected females may bite more frequently than uninfected individuals (Figure 3.3), with possible consequences for malaria transmission. However, as predicted by life history theory $^{32}$, an increase in fecundity would likely induce a compensatory decrease in longevity (not measured in our experimental design), a key component for the completion of the parasite cycle within the mosquito vector.
Figure 3.3 | *Wolbachia* infection is predicted to increase the frequency of blood feeding events. The timing of blood feeds was determined using an individual-based stochastic model with the length of each gonotrophic cycle estimated from the observed time of egg-laying (Figure 3.2d). Green and grey lines indicate *Wolbachia*-infected and *Wolbachia*-uninfected mosquitoes, respectively. Each curve represents the combination of one million replicate simulations. Assuming a daily adult mortality rate of 0.15 in both groups, the average number of bites over a lifetime is 1.38±1.34 for uninfected females and 1.56±1.57 for *Wolbachia*-infected females. Most mosquitoes bite less than twice in their life span but a few may bite as many as eight times.

**wAnga interferes with natural *Plasmodium* infections**

A crucial question concerning *Wolbachia* infections in *Anopheles* mosquitoes is whether these bacteria impact *Plasmodium* transmission by the mosquito vector. As Burkina Faso is a region of high malaria transmission, we reasoned that we could directly assess the effects of natural wAnga infection on the prevalence of *Plasmodium* parasites in blood-fed females collected from the interior of houses in the Vallée du Kou. We dissected a total of 221 blood-fed *An. coluzzii* females (genotyped post hoc) five days post-collection, a time when the blood meal is fully digested and oocyst development is underway. DNA was extracted from samples containing abdomens and thoraxes (encompassing the ovaries, the midgut, and the salivary glands) dissected from individual females to unravel a possible interaction between the presence of
Wolbachia and Plasmodium in these individuals. A total of 116 females were positive for wAnga, with an infection prevalence of 52.5% (116/221). Plasmodium infections were detected in 12 females, producing a prevalence of 5.4% (12/221), comparable to previously reported data for this region \(^{33}\). We found a strong bias for Plasmodium infection in Wolbachia-negative individuals; 11 Plasmodium-positive females were negative for Wolbachia, and only 1 female showed co-infection with bacteria and parasites (Fisher’s exact post hoc test on unnormalized data, two-tailed, \(p = 0.0018\)) (Figure 3.4a and Table S4). This highly significant reduction – over 90% – suggests that wAnga may interfere with Plasmodium development in the mosquito vector, as shown in artificial Anopheles-Wolbachia combinations \(^{16,15,21}\).

We went on to model whether natural Wolbachia infections would affect malaria transmission dynamics when these results, obtained on a limited sampling of a single transmission area, are extrapolated on a broader scale. We used a modified Ross-Macdonald model with additional compartments for Wolbachia-infected mosquitoes, which differ in their susceptibility to malaria (Figure 3.4a) and in the length of their gonotrophic cycle (Figure 3.2d), an effect that would likely impact their biting rates (Figure 3.3). We considered the Wolbachia-infected populations to have an identical mortality rate to uninfected mosquitoes (Figure 3.4b, red line) but also ran a model where the increased speed of the gonotrophic cycle is paralleled by a decrease in female lifespan (Figure 3.4b, blue line) as predicted by life history theory. Our model suggests that in both scenarios, infection with these bacteria would significantly decrease malaria prevalence in humans, even within the range of Wolbachia frequencies that we observed in mosquito populations.
Figure 3.4 | wAnga infections reduce malaria prevalence in mosquito and human populations. (a) Blood-fed females were collected from houses in Vallée du Kou, and allowed to develop eggs and oviposit. A minimum of 5 days post-collection, thoraxes and abdomens were dissected and screened for the presence of wAnga and Plasmodium by 16S (or 16S nested) PCR and 18S qPCR, respectively. Shown in red are the proportions of Plasmodium-infected females in both wAnga-infected (green, N = 116) and uninfected (grey, N = 105) females (Fisher's exact post hoc test on unnor malized data, two-tailed, p = 0.0018) (see Table S4). Numbers in parentheses indicate the sample size. (b) A modified Ross-Macdonald model incorporating Wolbachia-infected mosquito compartments was used to determine the impact of Wolbachia on malaria prevalence. In addition to variation in biting rate and susceptibility to malaria, Wolbachia-infected mosquitoes were either considered to have an identical daily mortality rate to uninfected mosquitoes (red line) or an enhanced daily mortality rate due to trade-offs with increased gonotrophic cycles (blue line). The shaded area represents the range in Wolbachia prevalence detected in our studies across multiple years.

wAnga infection in mosquitoes from lab-adapted colonies does not affect P. falciparum NF54 infections

To determine whether we could recapitulate the negative correlation we observed between Wolbachia and Plasmodium in natural populations of An. coluzzii in laboratory conditions, we conducted experimental P. falciparum infections of our laboratory wAnga-infected An. coluzzii mosquito line. Prior to infection, the mosquito line was enriched for Wolbachia by having females oviposit in individual cups, screening them for Wolbachia by 16S nested PCR, and pooling only the progeny of Wolbachia-positive females (called the wAnga-selected, or
wAnga-S line). Females from the wAnga-S lines were fed on *P. falciparum* NF54 gametocyte cultures, and 7-8 days post-infection their midguts were collected and stained individually to count the number of oocysts. The carcasses of each female were collected, and DNA was extracted to screen for wAnga infection by 16S nested PCR. The number of oocysts developed was then matched back to the *Wolbachia* infection status for each female. We found that wAnga infection did not impact the number of oocysts developed per female (Student’s t-test on log_{10}-transformed ratios, t = 0.3894, d.f. = 92, p > 0.05) (Figure 3.5a) nor the prevalence of infection (Fisher’s exact test, two-tailed, p > 0.05) (Figure 3.5b), in contrast to what we observed in natural *Wolbachia-Plasmodium* associations in Burkina Faso. However, few wAnga-infected females harbored high intensity infections, with most females developing fewer than 30 oocysts (Figure 3.5a). These results suggest ecological factors influencing *Wolbachia*’s negative effects on *Plasmodium* parasites in the field may be absent in laboratory infections. However, a caveat of these experiments is that the intensity of *Wolbachia* infections were often at the limit of our detection level, making it challenging to reliably select for a true *Wolbachia*-negative control group.
Figure 3.5 | \textit{wAnga infection does not affect} \textit{P. falciparum} \textit{infection intensity or prevalence in laboratory conditions}. Females from a laboratory-adapted \textit{wAnga}-infected colony were infected with \textit{P. falciparum} NF54, and 7-8 days post-infection their midguts were dissected and stained with mercurochrome to detect the presence/absence of infection and determine the number of oocysts developed per female. The carcasses (including the ovaries) of these females were screened for \textit{wAnga} infection by 16S nested PCR, and \textit{wAnga}-infection status was then matched back to individual oocyst counts. \textit{wAnga}-infected females are shown in green (N = 39) and uninfected females are in grey (N = 73) (a) \textit{wAnga} infection did not significantly affect the number of oocysts developed per female (Student’s t-test on log$_{10}$-transformed ratios, t = 0.3894, d.f. = 92, p > 0.05). Lines represent means with standard errors of the means. (b) The prevalence of infection (proportion of females infected with \textit{P. falciparum} NF54, in red) was not different in \textit{wAnga}-infected (green) and uninfected (grey) females (Fisher’s exact test, two-tailed, p > 0.05).

**DISCUSSION**

Our findings that natural \textit{Wolbachia} infections persist in \textit{Anopheles} mosquito populations and are negatively \textit{correlated} to \textit{Plasmodium} parasite development bring into sharp focus the potential impact of these bacteria on malaria transmission. Although our study is limited to samples from the VK5 village and we have not yet determined the effects of \textit{wAnga} on malaria transmission by other important vectors like \textit{An. gambiae} and \textit{An. arabiensis}, the observed negative correlation between \textit{Plasmodium} and \textit{Wolbachia} prevalence in \textit{An. coluzzii} suggests
that malaria dynamics may be affected by the stable presence of these endosymbionts. Indeed, a recent study detected Wolbachia infections of An. coluzzii in Mali and also reported that infection with this bacterium reduced P. falciparum infection. Provided our results are confirmed when additional samples, species, and geographical locations are tested, our mathematical model predicts that increasing Wolbachia prevalence would reduce malaria infections in the human population, even when incorporating an increase in biting rates and regardless of a possible trade-off in lifespan.

How can wAnga infections have such a significant impact on Plasmodium transmission? A number of studies have shown that strong anti-pathogenic effects likely caused by immune system activation are often associated with novel Wolbachia transinfections. Consistent with the previously determined imperfect maternal transmission rates and the lack of CI detected in this study, the observed interference with Plasmodium suggests that wAnga represents a recently introduced infection not yet fully adapted to the Anopheles host. Although we did not characterize localization of these bacteria in tissues besides the ovaries, we previously detected wAnga in An. coluzzii carcasses that excluded germline tissues. These results suggest wAnga also infects somatic tissues, including those where Plasmodium development occurs, possibly inducing parasite killing via the upregulation of the mosquito immune system.

As hypothesized in other Wolbachia infections, wAnga may also effectively compete with Plasmodium for nutrient resources. Following blood feeding, large amounts of lipid transporters circulate to transfer lipids from the gut and the fat body to the ovary. These same lipid transporters are required by Plasmodium to evade the mosquito immune system. Wolbachia’s potential diversion of resources may allow increased rates of immune system-mediated killing of Plasmodium. Alternatively, these endosymbionts may compete with other bacteria in the microbiome, especially following a blood meal, further draining resources away from developing Plasmodium parasites. Our data from laboratory infections showing that few
wAnga-infected females had high oocyst intensities could lend support to this hypothesis, as it suggests that wAnga could outcompete *P. falciparum* when resources become limiting, such as when parasitic infection is high.

Our ability to elucidate the mechanism whereby wAnga blocks *Plasmodium* development is complicated by our laboratory results showing no effect of these endosymbionts on *P. falciparum* infection intensity and prevalence. Such discrepancies in results collected from field vs. laboratory studies suggest that complex factors underlie natural *Wolbachia-Plasmodium* associations. High *Wolbachia* titers have been correlated to the strength of the pathogen-blocking effect of this bacterium \(^{35-37,41}\), but we have evidence suggesting that once wAnga-infected mosquitoes are transitioned from natural to laboratory settings, *Wolbachia* titers rapidly decline. While 16S PCR is often sufficient to identify *Wolbachia* infection in field mosquito samples, nested PCR is required to detect infection in laboratory colonies. Moreover, wAnga infection in our laboratory *An. coluzzii* colony has been difficult to maintain and requires periodic selection of the progeny of wAnga-infected mothers (as described above in the wAnga-S line). Therefore, while *Wolbachia* titers in natural anopheline populations may be high enough to confer resistance to *Plasmodium* infection, decreasing levels of this bacterium in laboratory-adapted colonies may no longer be sufficient to induce these protective effects.

In addition to *Wolbachia* titers influencing parasite development, ecological factors may maintain and shape these bacterial infections in natural populations of *An. gambiae* and *An. coluzzii*. Indeed, cyclical temperatures impact bacteria densities and *Wolbachia*-induced phenotypes \(^{42}\), while competition and survival in larval breeding sites are affected by infection with this endosymbiont \(^{43}\). Furthermore, features of the *P. falciparum* parasite itself may influence its susceptibility to wAnga’s effects. Our laboratory experiments examine infection with a single strain of *P. falciparum*, but it is possible the *An. coluzzii* mosquitoes assayed in our field experiments were infected with multiple parasite clones, a variable known to impact the mosquito’s immune
response to infection \cite{44} and which could shape *Wolbachia*’s effects on the parasite. These environmental factors, in addition to many other pressures including insecticide exposure, could be actively shaping and/or selecting for *wAnga* and impacting how it affects the *Plasmodium* parasite. Elucidating the ecological factors that may promote *Wolbachia*’s negative effects on *Plasmodium* in natural settings will be key to determining potential molecular mechanisms mediating this interaction.

Regardless of the mechanism, in future studies it will be crucial to test *Wolbachia*-*Plasmodium* interactions in different geographical locations (in addition to the villages tested in our study in Burkina and in the villages reported in Mali \cite{30}) and across different transmission seasons to determine the possible heterogeneity in pathogen-blocking effects across sub-Saharan Africa, a factor that would have consequences for the interpretation of the outcome of key malaria control strategies such as LLINs and IRS \cite{3}.

We found that *wAnga* infections cause limited reproductive phenotypes in the *Anopheles coluzzii* host, and in our mating assays we did not detect CI at significant levels. Although observed in multiple *Wolbachia*-infected arthropods, CI is not a universal phenomenon, and its presence and penetrance is highly dependent on *Wolbachia* strain and host organism. It remains to be established whether CI could be triggered by *wAnga* infection in species that do not naturally harbor these endosymbionts, as this would be an important first step towards integrating the use of *wAnga* into malaria control strategies. A host-dependent ability of *Wolbachia* to induce CI in non-natural hosts (reviewed in \cite{45}) has been demonstrated in many insects including the major dengue vector *Aedes aegypti*, in which the fruit fly *wMel* strain induces strong CI \cite{46} and has successfully invaded natural populations \cite{47}. Furthermore, *Wolbachia* densities, CI, and other reproductive phenotypes can also be modulated within the same host by physiological, ecological, and environmental factors including the age of the insect \cite{48}, temperature \cite{42,49}, and larval density \cite{43}. Finally, the recent discovery of proteins from the *Wolbachia* WO prophage that induce CI
phenotypes even in the absence of the *Wolbachia* bacterium \(^{11,12}\) provide us with the unprecedented capacity to introduce these genes into the *Anopheles* genome and determine whether CI can be induced in these important vector species.

Although *w*Anga did not increase the number of eggs laid by infected females, it increased the rate at which these eggs were laid. Over multiple gonotrophic cycles, this reproductive phenotype could increase lifetime female fecundity, although life history theory predicts that this effect would be counteracted by a trade-off with longevity \(^{32}\). The mechanism of this effect may relate to more efficient blood meal digestion, lipid deposition within the egg, egg maturation, activation of hormonal pathways that trigger oviposition \(^{50}\), or a combination of these factors. Furthermore, in conditions of nutrient deprivation, *w*Anga could be provisioning blood-fed females with additional nutrients as shown in other *Wolbachia* infection models \(^{51-53}\), ensuring timely oviposition. These hypotheses remain to be tested, and the colonized *Wolbachia*-infected *An. coluzzii* line may prove instrumental to determining the full extent of reproductive manipulations that *w*Anga inflicts on its mosquito host.

Combined with the previously reported identification in *An. gambiae* in the same region, the finding of *w*Anga infections in natural populations of *An. arabiensis*, a vector species capable of remarkable plasticity in its blood feeding and resting behavior, suggests that this natural *Wolbachia* strain is capable of adapting to different anophelines. *w*Anga therefore provides a promising new tool for future malaria control strategies aimed at exophilic and exophagic *Anopheles* species not targeted by current vector control strategies.
MATERIALS AND METHODS

Mosquito collections

Two *Anopheles* species were collected from two separate field sites near Bobo-Dioulasso, Burkina Faso. *An. coluzzii* were collected from the VK5 village (11°23’N; 4°24’W) in the Vallée du Kou, located 30 km northwest of Bobo-Dioulasso. Mosquitoes were collected in two ways: (1) blood-fed adult females were captured in houses in the village; (2) fourth instar larvae were collected from breeding sites in the rice fields surrounding the village and bred to adulthood in an insectary. *An. arabiensis* were collected from houses in the village of Soumousso (11°00’N; 4°02’W) located 55 km northeast of Bobo-Dioulasso as blood-fed adult females.

Establishment of a wAnga-infected *An. coluzzii* colony

wAnga-infected *An. coluzzii* mosquitoes were colonized by breeding eggs collected from VK5 to adulthood in an insectary. Adult females emerging from these eggs were crossed to *An. coluzzii* males from the Mopti colony (MR4) over 2 generations. At each generation, after egg-laying, infection in these hybrids was determined via PCR on *Wolbachia* 16S rDNA, and the progeny of wAnga-infected mothers was pooled to establish the line. A line with the same genetic background but cleared of wAnga infection was established by treating a subset of the F3 generation with tetracycline during larval development (1 µg/ml) and adulthood (10 µg/ml). The wAnga-infected *An. coluzzii* colony was periodically purified for *Wolbachia* infection by having females oviposit in individual cups, screening them for *Wolbachia* by 16S nested PCR, and pooling only the progeny of *Wolbachia*-positive females.

DNA extraction and mosquito species genotyping

Mosquitoes were beheaded and the genomic DNA of carcasses was extracted using the Qiagen Blood and Tissue kit (Qiagen) with an extended lysis incubation step. In brief, carcasses were
homogenized in 1X phosphate-buffered saline (PBS) and incubated with the kit’s proteinase K and AL lysis buffer for 30 minutes at 56°C. The remaining extraction steps were performed following the kit’s supplementary protocol for DNA extraction of insect cells. For An. coluzzii, An. gambiae, and An. arabiensis genotyping, the S200 x 6.1 locus from genomic DNA was amplified by PCR using standard protocols and with the following primers: FWD 5’-TCGCTTAGACCTTGCTTA-3’, REV 5’-CGCTTCAAGAATTGGATAC-3’ 54.

**wAnga detection by PCR and sequencing**

Detection of wAnga infection in mosquito carcasses was performed by PCR amplification of the 16S rDNA region using *Wolbachia*-specific primers (W-Specf: 5’-CATACCTATTGGAAGGATAG-3’, W-Specr: 5’-AGCTTCAAGGAAACCAATTC-3’) and standard protocols 55. In cases of low infection intensity, nested PCR was used for wAnga detection. A total of 2 µl of the amplification product from the initial 16S rDNA PCR was amplified using specific internal primers (16SNF: 5’-GAAGGGATAGGGTGTCGTCG-3’, 16SNR: 5’-CAATTCCCATGCGCGCTGTGACG-3’) and HotStarTaq (Qiagen) in a 20 µl reaction volume. The nested 16S rDNA PCR cycling conditions used were 15 minutes at 95°C, followed by 35 cycles of 15 seconds at 95°C, 25 seconds at 66°C, and 30 seconds at 72°C, followed by 5 min at 72°C. The sequence of the resulting 412 bp fragment was determined by Sanger sequencing. All samples sequenced were confirmed to correspond to *Wolbachia* (Figure S7), demonstrating that the nested PCR protocol did not generate any false positives.

**wAnga detection in mosquito oocytes by FISH**

DNA probes specific to wAnga 16S rDNA were designed and synthesized (5’-Cy3-CGAGGCTAAGCTAATCCGTAAA-3’, Integrated DNA Technologies). The ovaries of wAnga-infected and tetracycline-treated females from the established wAnga colony were dissected in 1X PBS, fixed in 4% paraformaldehyde, and treated with 80% ethanol-6% hydrogen peroxide to
remove autofluorescence. FISH was then conducted using a protocol modified from Toomey *et al.* 2013. Following initial pre-hybridization equilibration steps, hybridization was conducted by incubating tissues with 1 ng/µl of the RNA probe in Hybridization solution containing 50% formamide, 5X SSC, 250 µg/ml salmon sperm DNA, 0.5X Denhardt’s solution, 20 mM Tris-HCl and 0.1% Tween at 37°C for 3 hours. Probe competition hybridization was conducted by incubating tissues with a mixture of the labeled RNA probe (1 ng/µl) and an unlabeled probe of identical sequence (20 ng/µl). Following washes in a solution containing 1X SSC, 0.1% Tween, 20 mM Tris-HCl then in a solution with 0.5X SSC, 0.1% Tween, 20 mM Tris-HCl at 55°C, tissues were blocked in 1% BSA in 1X PBS-0.1% Tween (PBST) and stained with 1:100 mouse anti-Cy3 antibody (Santa Cruz Biotechnology). Tissues were then incubated in 1:1000 goat anti-mouse Alexa488 antibody (ThermoFisher Scientific) and in 1µg/ml DAPI (Sigma-Aldrich). All staining steps were followed by washes in PBST. Tissues were then mounted in Vectashield mounting media and images were acquired on a Zeiss Axio Observer inverted fluorescent microscope with Apotome2. Post-imaging processing was done using ImageJ and Adobe Photoshop CS5.

**Analysis of reproductive phenotypes**

For cytoplasmic incompatibility (CI), fecundity, and sex ratio analyses, mosquitoes were collected as fourth instar larvae from breeding sites or as eggs deposited by blood-fed females collected from houses in VK5 in the Vallée du Kou. These field-collected larvae and eggs were bred to adulthood in the insectary, and adult males and females were crossed in single pairings by forced mating (protocol available on [https://www.beiresources.org/MR4Home.aspx](https://www.beiresources.org/MR4Home.aspx)). DNA from males was immediately extracted and wAnga infection status was determined by 16S PCR. Females were blood fed and placed into individual cups to allow them to oviposit individually. Following oviposition, the genomic DNA of these females was extracted and screened for wAnga infection by 16S PCR. Egg broods were scored for inviability (a direct indicator of CI), and the number of eggs laid (fecundity). Females were also dissected to determine the number of eggs retained and
consequently the total number of eggs developed. To assess the sex ratio of the progeny, broods from these crosses were bred to adulthood and the number of males and females in each brood was determined. Infection status of males and females in each cross was determined post hoc by 16S PCR.

For timing of oviposition, blood-fed females were collected from houses in VK5 and put into cups 2 days post-blood-feeding to allow them to oviposit individually. Oviposition time was monitored by checking for the presence of eggs in each cup every day following provision of an oviposition site.

**Plasmodium detection by qPCR**

The quantitative PCR protocol published by Bass et al. 2008 for *Plasmodium* detection was modified for use with SYBR green dye. Genomic DNA extracted from female carcasses was 10-fold diluted, and 5 µl of this dilution was quantified in a mix with 1X Fast SYBR® Green Master Mix (ThermoFisher Scientific) and 300 nM of each primer targeting a region in the *Plasmodium* 18S rDNA sequence (PlasF: 5’-CTTAGTTACGATTAATAGGAGTAGC-3’, PlasR: 5’-GAAAATCTAAGAATTTCACCTCTGA-3’) in duplicate reactions on a StepOnePlus Real-Time PCR System (Applied Biosystems). Relative quantities were calculated using a standard curve built with serial dilutions of a plasmid containing the targeted 18S rDNA sequence. This plasmid was made by PCR amplifying the 18S rDNA region of genomic DNA extracted from the *P. falciparum* P2G12 strain with the PlasF and PlasR primers, and cloning the resulting product using the pGEM®-T Vector System (Promega). Copy number quantification was determined using a Nanodrop 2000c Spectrophotometer (ThermoFisher Scientific).

**Experimental infections of wAnga-infected mosquito colonies with *P. falciparum***

All cultures were maintained at 37˚C in an incubator gassed to 5% oxygen, 5% carbon dioxide, 90% nitrogen. Asexual cultures of NF54 were maintained below 2% parasitemia at 5% hematocrit
(O+ red blood cells, RBC) in complete medium (RPMI 1640 with L-glutamine and 25 mM HEPES, 10 µg/ml hypoxanthine, 0.2% sodium bicarbonate, 10% O+ human serum). To induce gametocytogenesis, cultures were grown without splitting to parasitemias of 3-10%, then split to 2% parasitemia in complete media at 5% hematocrit (using less than 1-week old RBC) to a total volume of 15 ml. 5 ml of this culture was aliquoted to 3 separate wells of a 6 well plate and cultured for 14-20 days, changing the media every day. To determine the exflagellation rate of a gametocyte culture to assess its suitability for an infectious feed, an equal amount of culture was taken up from each of the three induction wells to a final volume of 400 µl and spun down at 1500 rpm for 1 minute at room temperature. 11.5 µl of the infected RBC pellet was added to 10 µl of human serum heated to 37˚C, resuspended, and incubated on a slide with a coverslip at room temperature for 15-20 minutes. Number of exflagellations per field was determined by microscopy at 100X for five fields to determine approximate infectivity of a culture. Gametocyte cultures with exflagellation rates greater than 10 were used for mosquito infections.

For the infectious feed, gametocyte cultures were spun down at 37˚C at 1800 rpm for 2 minutes, then diluted 1:10 in a 1:2 mix of fresh RBC and human serum at 37˚C. 160 µl of these samples was loaded into custom-designed Chemglass Life Sciences glass membrane feeders sealed with parafilm and heated to 37˚C with a Haake D1 Immersion Circulator, allowing the feed of experimental and control groups simultaneously on the same gametocyte culture. Mosquitoes were allowed to feed for 30-60 minutes, adding more culture as necessary, and all non- and partially-fed females were removed from cages following the infection using a Bioquip ethanol aspirator.

7-8 days post-infection, the midguts of mosquitoes were dissected and stained in 2 mg/ml mercury dibromofluorescein disodium salt (mercurochrome) for 12-15 minutes, then loaded onto 10-well slides in 0.2 mg/ml mercurochrome and covered with a coverslip for visualization and counting by microscopy.
Model of feeding time

Individual female mosquitoes were simulated stochastically through the course of their adult life. Upon emerging as adults, all female mosquitoes rested for one night prior to mating. The following night females took their initial blood meal, beginning gonotrophic cycles, which consisted of feeding, resting for up to three nights and egg laying. The number of nights spent resting prior to egg laying by each female in each gonotrophic cycle was determined probabilistically from the data on oviposition (Figure 3.2d). This process was simulated in MATLAB R2015a (The MathWorks, Inc., Natick, MA, USA) for one million mosquitoes to determine what proportion of mosquitoes fed each night.

Malaria transmission model

The Ross-Macdonald compartmental model of malaria transmission was adapted to include Wolbachia-infected mosquitoes, forming a system of five delay differential equations. The infected human population ($I_H$) grew when susceptible humans received infectious bites and declined as humans recovered at a fixed rate ($r=0.05$). Mosquito populations ($E_M, E_W$) became infected after an infectious bite but were not infectious ($I_M, I_W$) until the completion of the latent period, with Wolbachia-infected populations denoted with a $W$-subscript. The fraction of the mosquito population infected with Wolbachia ($W$) was constant throughout a single simulation.

\[
\frac{dI_H(t)}{dt} = abmI_H(t)[1-I_H(t)] - rI_H(t)
\]

\[
\frac{dE_M(t)}{dt} = acI_H(t)((1-W) - E_M(t) - I_M(t)) - acI_H(t-\tau)(1-W) - E_M(t-\tau) - I_M(t-\tau)]e^{-\tau} - uE_M(t)
\]

\[
\frac{dI_M(t)}{dt} = acI_H(t-\tau)((1-W) - E_M(t-\tau) - I_M(t-\tau)]e^{-\tau} - uI_M(t)
\]

\[
\frac{dE_W(t)}{dt} = \hat{a}\hat{c}I_H(t)[W - E_W(t) - I_W(t)] - \hat{a}\hat{c}I_H(t-\tau)[W - E_W(t-\tau) - I_W(t-\tau)]e^{-\tau} - \hat{u}E_W(t)
\]

\[
\frac{dI_W(t)}{dt} = \hat{a}\hat{c}I_H(t-\tau)[W - E_W(t-\tau) - I_W(t-\tau)]e^{-\tau} - \hat{u}I_W(t)
\]
The Wolbachia-infected and uninfected mosquitoes differed only in biting rate \((a=0.5, \hat{a}=0.565)\), susceptibility to malaria infection \((c=0.79, \hat{c}=0.07)\), and daily mortality rate \((\mu=0.15^{60-62} \text{ red line}, \hat{\mu}=0.175 \text{ blue line in Figure 3.4b})\). The probability of transition to humans \((b=0.4^{58})\), the length of the latent period \((\tau=12^{63})\), and the relative mosquito to human ratio \((m=7.7^{58})\) were identical between both mosquito populations. Hats denote parameters associated with Wolbachia-infected mosquitoes. The biting rate \((\hat{a})\) was enhanced by the relative number of blood feeds possible by Wolbachia-infected females compared to Wolbachia-uninfected females as determined in our feeding model, \(\hat{a}=1.13a\) (Figure 3.3). The susceptibility of Wolbachia-infected females to malaria infection \((\hat{c})\) was discounted based on the data presented in Figure 3.4a, \(\hat{c}=c/11\). The mortality rate \((\hat{\mu})\) was increased (Figure 3.4b, blue line) to impose a fitness costs on Wolbachia-infected females, such that the average number of feeds was identical for Wolbachia-infected and uninfected mosquitoes. All simulations were performed in MATLAB R2015a (The MathWorks, Inc., Natick, MA, USA) using the dde23 solver.

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CHAPTER 4:

Discussion

This chapter was written by Perrine Marcenac, with editorial input from Flaminia Catteruccia.
4.1 Overview

There is a wealth of evidence that *Plasmodium* has exerted strong selective pressure on the human genome \(^1\); however, few studies have examined to what extent this parasite has shaped the genome of its definitive host, the *Anopheles* mosquito. Although transmission of the deadliest malaria parasite, *Plasmodium falciparum*, began relatively recently, likely within the last 100,000 years \(^2\)–\(^4\), the burden of ancestral forms of these parasites on their mosquito vectors has been significant over millions of years. There is natural, geographic heterogeneity in the level of that burden among the anophelines that are competent to transmit this parasite across the globe. Species of the subgenus *Cellia* that encompass the African and Asian vectors of malaria are in areas with highest rates of *P. falciparum* transmission, while the New World vectors of the subgenus *Nyssorhynchus* have only been infected within the last 500 years \(^5\). Evidence of selective pressure on the *Anopheles* genome may therefore be found by examining mosquito traits that are relevant for vectorial capacity and have diverged between vectors populating “ancient” versus “recent” malaria areas.

In an attempt to identify mosquito factors that may have been affected by evolutionary pressures imposed by *Plasmodium* on the mosquito genome, we focused our attention on reproductive traits that influence egg development after blood feeding and have diverged between Old and New World species, namely the cascade of events triggered in the female mosquito by sexual transfer of the steroid hormone 20-hydroxyecdysone (20E). Based on our own meta-analysis showing the parasite does not reduce fecundity in its natural African vectors (Chapter 1, Figure 1.7), we hypothesized that *Anopheles* species that have experienced significant pressure from *P. falciparum* have evolved tolerance mechanisms to infection. Moreover, in a separate study, we examined whether ecological factors can influence the *Anopheles-Plasmodium* interaction and shape female tolerance and resistance to the parasite. Here, we revisit the major findings of this dissertation and place these results in the context of other works on vector-parasite
interactions. Furthermore, we identify extant questions and future directions of this work as we continue exploring the evolution of the Anopheles-Plasmodium interaction.

4.2 The female protein MISO confers tolerance to P. falciparum infection in Anopheles gambiae

The sexual transfer of the steroid hormone 20E is a divergent trait that has evolved in Old World species of the Cellia subgenus, including Anopheles gambiae, Anopheles arabiensis, Anopheles funestus, Anopheles dirus, Anopheles farauti, and Anopheles stephensi, but is notably absent in the Nyssorhynchus species Anopheles albimanus (Figure 1.6). In An. gambiae, transfer of this hormone influences blood feeding processes by increasing egg production post-mating via the gene Mating-Induced Stimulator of Oogenesis (MISO). MISO is induced post-mating in the atrium in a 20E-dependent manner, forms a complex with 20E, and impacts oogenesis possibly by boosting production of the lipid transporter Lipophorin (Lp) post-blood feeding. According to our previous analyses, it is likely that transfer of male 20E during mating has driven the reciprocal adaptation of MISO. These studies demonstrated that the function of a female protein bridges two processes essential to An. gambiae reproductive fitness – mating and blood feeding. Given the remarkable overlap between the geographical distribution of the species that transfer 20E during mating and the areas of greatest P. falciparum transmission, our hypothesis was that the evolution of this reproductive trait may have impacted vectorial capacity.

Indeed, as detailed in Chapter 2, we reveal that MISO confers tolerance to An. gambiae females infected with P. falciparum. Silencing of this gene induced a fitness cost by reducing the fecundity of infected females in the context of both laboratory (Figure 2.2) and field infections (Figure 2.3), but it did not impact parasite intensity or prevalence. We identified that in addition to being sensitive to 20E signaling in the atrium post-mating, MISO is produced in the ovaries post-blood feeding in a 20E-dependent manner (Figure 2.5) and localizes to the oocyte within the ovarian follicle and in the trachea attached to the ovaries (Figure 2.6). As a mechanism
whereby MISO maintains female reproductive fitness in the face of *P. falciparum* infection, we find that it is not involved in preventing apoptosis in ovarian follicles (Figure 2.8), but rather may be important in transducing hypoxia-induced factor (HIF) signaling towards proper ovarian growth and egg development (Figure 2.7). Specifically, expression of the HIF transcription factor HIF\(\beta\) and the HIF\(\alpha\)-inactivating prolyl hydroxylase *fatiga* was increased post-blood feeding and at timepoints post-mating in reproductive tissues, but MISO silencing dampened the transcription of these genes both in the context of mating and blood feeding (Figure 2.7). We also uncovered that the key molting neuropeptide hormone *Ecdysis-Triggering Hormone* (ETH) is included in putative co-regulated factors based on computational analysis on microarray datasets (Table 2.2 and Figure 2.4).

MISO provides the first example of a reproductive mosquito factor mediating tolerance to *P. falciparum* infection. While in these experiments we focused on fecundity (the total number of eggs developed) as the metric for reproductive fitness, future experiments will also need to examine the quality of the offspring produced by assessing fertility of broods following infection. Furthermore, we found that components of the HIF signaling pathway are induced by blood feeding, and that MISO may play a role in transducing this signal. Hypoxia and hypoxia-induced signaling has yet to be investigated in *Anopheles* species, but our results suggest it acts within 20E-induced pathways and may be important for egg development. This is supported by recent studies demonstrating a link between hypoxia and 20E signaling in *Aedes aegypti* ⁸,⁹, though these works focused on larval, rather than egg, development. These results open exciting new avenues of research, as it is unlikely MISO acts alone to induce tolerance to malaria infection given its lack of functional domains and structure, and its function within HIF signaling or in relation to ETH may be the key to the fitness-conferring phenotype we observe in *An. gambiae*.

To investigate this question, ETH’s role in *An. gambiae* will need to be elucidated, as no studies have been published on this hormone in this species. Furthermore, although a link between ETH and 20E has yet to be determined in *Anopheles*, two observations suggest the two
hormones may somehow be working in the same pathways: in some insects ETH is critical to larval ecdysis \textsuperscript{10,11} and it ensures normal egg development \textsuperscript{12}, and both these biological processes are mediated by 20E. MISO’s sensitivity to 20E and its presence at high levels in the trachea (Figure 2.6), the tissue sensitive to ETH signaling, suggest that MISO and ETH may be molecularly or functionally linked. Studies silencing \textit{ETH} will help determine its contribution to \textit{An. gambiae} oogenesis and whether it functions with MISO, potentially elucidating new molecular factors contributing to anopheline reproductive fitness.

The role of HIF signaling post-mating and post-blood feeding will need to be investigated in greater depth. During larval development in other insects, tissues become hypoxic and initiate a hypoxic transcriptional program when larvae grow to proportions that the fixed tracheal system can no longer adequately oxygenate, triggering ecdysone production and consequently ecdysis \textsuperscript{13}. A key aspect of future studies will be to understand whether our data demonstrating that components of HIF signaling are induced post-blood feeding results from the ovaries experiencing a similar “molting-like” response. Could local oxygen levels in the ovaries drop as eggs begin to develop and grow because the tracheal system associated with the ovaries cannot accommodate the increasing size, in turn triggering production of ecdysteroids that are essential for oogenesis to proceed? Very little is known on the molecular pathways that induce ecdysone and 20E production in \textit{Anopheles} mosquitoes post-blood feeding, and so these studies have the potential to uncover signaling factors important for egg development in these species. To begin investigating these questions, measuring oxygen levels in the ovaries, as well as determining potential effects of promoting or blocking HIF signaling (via treatment with the prolyl hydroxylase inhibitor FG-4592 or the HIF\(\alpha\) inhibitor PX-478, respectively) on fecundity following infected and uninfected blood meals will be a critical next step. Furthermore, to determine whether there is a link between HIF and 20E signaling post-mating and post-blood feeding, as our results suggest, we can silence the 20E nuclear receptor \textit{Ecdysone Receptor} (EcR) and assess transcription of
factors involved in HIF signaling, and also measure 20E levels in females with impaired HIF signaling.

Could *P. falciparum* modulate HIF signaling within *An. gambiae*? To answer this question, we will need to elucidate how blood feeding triggers a hypoxic transcriptional response. HIF signaling could be induced by the production of reactive oxygen species (ROS), or via expansion of the midgut microbiome post-blood feeding. Moreover, *P. falciparum* could increase ROS produced in the midgut, or it could alter components in the blood meal (such as increased free heme or bilirubin) in order to elicit a stronger hypoxic response. Determining whether these components in the blood are altered in *P. falciparum*-infected samples, and whether HIF signaling is increased following an infectious blood meal relative to an uninfected feed, may provide clues into a possible link between HIF signaling and parasite development. Demonstrating that *P. falciparum* infection influences the hypoxic response would provide further evidence that the parasite can manipulate the mosquito environment for its own benefit, beside its already known capacity to exploit lipid transporters produced downstream of 20E signaling post-blood feeding.

4.3 Investigating possible effects of immunity on *Anopheles* tolerance to *P. falciparum*

Previous studies have posited that *Pfs47* haplotypes allow *P. falciparum* to adapt to local vectors and thus silence the mosquito immune response, but only in local vector-parasite associations. This hypothesis has been experimentally supported in *An. gambiae* infected with the laboratory-adapted African *P. falciparum* strain NF54, where silencing of the gene *thioester-containing protein 1* (*TEP1*), a major modulator of the mosquito immune response to *Plasmodium*, did not affect oocyst prevalence or intensity, but a *Pfs47* knock-out NF54 strain was highly susceptible to the mosquito immune system and TEP1-mediated killing. Our own data from laboratory infections of *An. gambiae* with NF54 support these studies, as we did not detect a fitness reduction in infected females, suggesting no costly immune response is mounted that

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4.3 Investigating possible effects of immunity on *Anopheles* tolerance to *P. falciparum*
could induce reproductive trade-offs. However, an outstanding question from our studies is whether we would indeed detect a reproductive cost to infection following activation of the immune system, as is the case when this species is infected with *P. falciparum* strains isolated from different geographical regions (such as with the American strain 7G8) or with a *Pfs47* knock-out NF54 strain. Future studies will require closer examination of potential reproductive trade-offs to infection with these other *P. falciparum* strains to determine whether tolerance mechanisms through MISO are also reliant on dampened mosquito resistance mechanisms mediated by Pfs47.

Our data studying infection in different anopheline species has demonstrated that the Central American vector *An. albimanus* suffers from reduced egg development when fed on a *P. falciparum*-infected blood meal. At this time, it is impossible to say whether this reduced tolerance to infection is due to the absence of the 20E-MISO interaction post-mating in this species, as previous studies have shown that *An. albimanus* mounts an immune response to *P. falciparum* NF54 that can be reversed by silencing of the immune protein LRIM1 involved in the complement-like response to infection. It will be crucial to determine whether potential trade-offs associated with an immune response following infection causes reduced tolerance phenotypes in *An. albimanus*, and whether similar costs to fecundity occur in the case of infections with the geographically-paired *P. falciparum* strain 7G8, which is less sensitive to *An. albimanus* immune insult.

Future studies will also focus on elucidating the role of MISO in *Anopheles* species in which males do not produce and transfer 20E during copulation, as MISO orthologues can be found in all anophelines as well as in *Aedes* and *Culex* species. Increasing amounts of evidence from studies in *Drosophila* and *Aedes* show that developmental pathways stimulated by 20E during molting are utilized in the adult stages to stimulate egg development. Given evidence that MISO may act within conserved hypoxia-induced, and potentially molting-related (through both HIF and ETH) signaling pathways, this protein could play a more ancestral role in species lacking sexually-transferred 20E. Species receiving 20E in the plug in turn saw MISO gain new function
to mediate the link between mating and blood feeding, as demonstrated in \(^6,7\) and in Chapter 2. Understanding the role of MISO across different anophelines will also potentially help us uncover which MISO in \textit{An. gambiae} – the protein produced in the atrium post-mating or the protein produced in the trachea and ovaries post-blood feeding – is responsible for the tolerance-conferring effects to \textit{P. falciparum}. RNAi silencing experiments demonstrated that while MISO knock-down is highly efficient at both the transcriptional and protein level in the atrium post-mating, it does not consistently reduce protein levels in the ovaries/trachea post-blood feeding (\textbf{Figure S3}). The fact that dsMISO females have reduced tolerance to \textit{P. falciparum} despite not consistently detecting reduced protein levels in the ovaries suggests the fitness phenotype we observe is at least partially due to MISO produced post-mating. These results, however, do not exclude the possibility that sexually-transferred 20E and production of MISO in the atrium post-mating primes the ovaries to be more responsive to 20E and MISO produced post-blood feeding, and so in this way both atrial and ovarian/tracheal MISO could contribute to tolerance to infection. Future work with the \textit{MISO} knock-out line currently being generated combined with functional studies of this protein in other \textit{Anopheles} species with and without sexually-transferred 20E will help clarify whether MISO's role in the ovaries/trachea or atrium or both mediates \textit{P. falciparum} tolerance phenotypes.

\textbf{4.4 African \textit{Anopheles} and infections with other human and ape malarias}

Given our results demonstrating that sexual transfer of 20E mediates tolerance to \textit{P. falciparum} infection through MISO in \textit{An. gambiae}, could pressure from \textit{Plasmodium} infection over millions of years have selected for the evolution of this male reproductive trait? Multistate ancestral reconstructions of male 20E and plug transfer in anopheline species estimate that the capacity to produce 20E in the MAGs arose after the \textit{Anopheles-Cellia} split approximately 60 million years ago (\textbf{Figure 1.6}) \(^6,18\). The \textit{Cellia} species which are distributed in sub-Saharan African, India, South-East Asia, and parts of Oceania may have experienced more pressure from
Plasmodium parasites; however, it is still unknown if all these species have mating-transferred 20E and/or suffer little costs to Plasmodium infection. To provide more insight into these questions, future research will need to examine other natural Anopheles-Plasmodium interactions, including P. falciparum infection in the South East Asian vector An. dirus, in the northern Oceanic vector An. farauti, in the Asian vector Anopheles sinensis, and in the South American vector Anopheles darlingi.

Sub-Saharan Africa has the highest rates of P. falciparum transmission in the world, but it is also home to a number of Plasmodium species infecting apes. Indeed, a 2010 survey of fecal samples from apes revealed widespread Plasmodium infection of western gorillas and chimpanzees, with most strains grouping within the Laverania radiation that include P. falciparum and the chimpanzee parasite Plasmodium reichenowi, and with many apes carrying multiple Plasmodium strains. These studies demonstrated that human P. falciparum originated from transmission of Plasmodium from gorillas. Recent reports point to three primary sylvatic species that transmit ape Plasmodium: Anopheles vinckeii, Anopheles moucheti, and Anopheles marshallii, all within the Cellia subgenus. Given that apes have complex infections with Plasmodium species of the Laverania subgenus and that the origin of P. falciparum is from these infections, these sylvatic vectors are interesting candidates to evaluate for reproductive fitness costs to infection as they are likely to have experienced massive, long-term pressure from Plasmodium. Moreover, it is reasonable to assume that these anopheline species also possess a 20E-based mating system, like all Cellia vectors of human malaria analyzed thus far. Another aspect to consider is whether An. gambiae (and other African vectors) would suffer a fitness cost to infection with ape Plasmodium species, as the An. gambiae lineage precedes agricultural modification of the environment, and so it is quite possible this species shared an ecological niche with apes and could have naturally transmitted these malaria species. Significant experimental hurdles will have to be overcome in these studies, as there are no laboratory colonies of sylvatic malaria vectors, nor are any of these ape malarias in culture. However, they
are an exciting avenue to consider in future studies, as they would provide invaluable insights into vector-parasite interactions and the evolution of molecular processes within the mosquito that can benefit the parasite.

Another interesting area of research as we try to understand whether the 20E-based mating system was selected for by *Plasmodium* infection is to determine whether *Anopheles* suffer a fitness cost to another major human malaria parasite, *Plasmodium vivax*. This parasite has a global distribution, with highest prevalence in South-East Asia\(^1^9\), while in sub-Saharan Africa human infection rates are low due to near fixation of a mutation\(^2^2\) that leads to the absence of the Duffy antigen receptor for chemokines (DARC) on the surface of erythrocytes, a receptor that is used by the parasite to invade red blood cells\(^2^3,2^4\). Recent studies point to an African origin for human *P. vivax*\(^2^5\), and apes in sub-Saharan Africa are infected with *P. vivax*-like parasites\(^2,2^5,2^6\). Furthermore, the last common ancestor of human *P. vivax* strains from different geographical areas is estimated to have existed over 700,000 years ago\(^2^7\), so it is likely that *Anopheles* have experienced long-term pressure from this parasite, particularly among African species. Intriguingly, a report estimates that *P. vivax* was first transmitted in the New World in pre-Columbian times, potentially 15,000 years ago\(^2^8\), and as a result examining the *P. vivax-An. albimanus* association could add another element to our studies of the evolution of tolerance mechanism. However, African vectors may still have experienced greater pressure from *Plasmodium* than American vectors, as there are numerous species of ape malaria in Africa, whereas in the Americas the *Plasmodium* species infecting monkeys are believed to have been the result of human-to-monkey transmission\(^2^8,2^9\). Without a reliable culture system for *P. vivax*, studying reproductive fitness costs in *Anopheles* will be difficult, but examining natural associations in the field may provide some insight into these questions.
4.5 *Wolbachia*’s effect on *P. falciparum* could help maintain *An. gambiae* tolerance

As discussed in greater detail in Chapter 1, in addition to examining the genetic components of tolerance and resistance, we must also consider how the ecological and environmental context of specific *Anopheles-Plasmodium* associations shape these interactions. Indeed, the *Anopheles* microbiome can significantly affect resistance mechanisms to *P. falciparum* by stimulating the transcription of anti-parasitic immune factors, including antimicrobial peptides, serine proteases, pattern recognition receptors, and peptidoglycan recognition proteins\(^30,31\). In Chapter 3, we report that infection with an *Anopheles*-specific strain of *Wolbachia* (*wAnga*) in field populations of *Anopheles coluzzii* negatively correlates with *Plasmodium* loads in individual mosquitoes (Figure 3.4); however, our laboratory infections did not demonstrate anti-parasitic effects on *P. falciparum* (Figure 3.5). These data suggest that *wAnga*’s resistance to these parasites may be environment-dependent, and a major future direction of this project will be to determine the mechanism underlying this difference.

Two plausible explanations are currently proposed that could explain how *Wolbachia* confers resistance to *Plasmodium* in *An. coluzzii*: 1) upregulation of the immune system and 2) resource competition between the pathogen and the bacteria. Upregulation of the immune system is more often associated with non-natural *Wolbachia*-insect associations, such as in the context of *Ae. aegypti* infections\(^32-34\) or with transinfected *Anopheles* species\(^35-37\). Furthermore, in studies on *Ae. aegypti* infected with the *Drosophila melanogaster Wolbachia* strain *wMel*, while some immune factors were upregulated, the authors did not detect consistent stimulation of factors within classical innate immunity signaling pathways\(^32\). The resource competition theory stems in large part from observations that in natural *Wolbachia*-insect associations, high titers of the bacteria associate with a greater anti-pathogenic effect\(^38-40\). Moreover, in the non-natural *Ae. aegypti*-*wMel* association\(^32\), as well as in high titer *wAlbB* infections of its natural host *Aedes albopictus*\(^40\), dengue virus was primarily found in cells that lacked *Wolbachia*. While it could be argued that higher titers lead to higher immune activation, it would be costly for the endosymbiont
to upregulate immune signaling, either constitutively or following blood feeding, in natural Wolbachia-insect associations, suggesting that resource competition within tissues that harbor both pathogen and bacteria may play a primary role in anti-pathogenic effects.

Our studies provide some support to the hypothesis that the effects wAnga has on Plasmodium are due to resource competition, although they do not exclude immune priming as a possible mechanism. First, for resource competition to occur, Wolbachia and Plasmodium would need to co-localize within tissues. Although we did not characterize localization of these bacteria in tissues besides the ovaries, we previously detected wAnga in An. coluzzii carcasses that excluded germline tissues, suggesting these bacteria could co-exist with Plasmodium in tissues like the midgut or salivary glands that are infested with parasites. Secondly, wAnga did not induce reduced fertility (Figure 3.2a) or fecundity (Figure 3.2b) in An. coluzzii females, indicating that bacterial infection does not cause a reproductive fitness cost as we may have observed had the immune system been upregulated. Furthermore, we have evidence that these bacteria can impact blood feeding processes, as infected females have accelerated gonotrophic cycles (Figure 3.2d). These data suggest Wolbachia can modulate host signaling post-blood feeding, strengthening the argument that it can reduce Plasmodium infection through resource competition. There is already a wealth of evidence indicating that Plasmodium relies on lipid transporters produced post-blood feeding for its own development, enabling lipid provisioning to the parasite. These data suggest that this parasite may be particularly susceptible to competition for lipids, and infection with wAnga could increase this competition. In support of this hypothesis, while we did not detect an effect of wAnga infection on P. falciparum in the laboratory, we did observe that Wolbachia-infected females tended to have fewer high-intensity infections (Figure 3.5a), suggesting that under conditions of high parasite burden where lipid resources may become more limiting, these bacteria can successfully out-compete the parasite.

Future experiments will need to more definitively assess whether resource competition, immune signaling, or both mediate the anti-plasmodial activity of wAnga. Specifically, gene
expression analysis of immune modulators, as well as experiments where wAnga-infected mosquitoes are assessed for *P. falciparum* infection in conditions where resources may be limiting will provide significant insight into the mechanism underlying *Wolbachia*’s effect on *Plasmodium*. Should the resource competition model prove to mediate *Plasmodium* killing, it would suggest that *Wolbachia* can confer resistance to *P. falciparum*, potentially at minimal cost to the vector. We have determined that neither the parasite (Chapter 2) nor wAnga (Chapter 3) induce a reproductive cost to *An. gambiae*. As such, these organisms may utilize a pool of resources that does not interfere with *An. gambiae* fitness, but that requires them to compete with one another for access to these resources in the case of co-infection.

4.6 Concluding remarks

Our studies have investigated the interaction of *P. falciparum* with its natural vectors, with a particular focus on its major vector *An. gambiae*. We have shown that *P. falciparum* benefits from a mating system utilizing the sexual transfer of 20E and the induction of the female protein MISO to ensure anopheline fitness in the face of infection. We have also found that in *An. coluzzii*, natural *Wolbachia* infections confer resistance to *Plasmodium*, suggesting the vector can utilize ecological factors to manipulate parasite survival without affecting its own tolerance to infection. The many questions highlighted in this chapter deserve consideration and further investigation in order to address the hypothesis that *Plasmodium* parasites could have driven the evolution of a 20E-based mating system, and that more specifically, *P. falciparum* could have exerted pressure on the *Anopheles* genome and selected for MISO’s function in conferring female tolerance to infection.

4.7 References


Ramasamy, R. Zoonotic malaria - global overview and research and policy needs. Front Public Health 2, 123 (2014).


APPENDIX A:

Supplementary Information for Chapter 2

Figure S1 | Virgin and mated Anopheles gambiae ovaries produce the same level of MISO post-blood feeding. 10 larvae in the L4 stage, 10 pupae, and 10-15 ovary pairs (with associated trachea) from mated and virgin females were collected before blood feeding (non-blood-fed, NBF), and 6, 12, 24, 36, and 60 hours post-blood feeding (hpbf). Protein samples were probed for MISO, and no difference was detected in the level of MISO produced in the ovaries/trachea of virgin and mated blood-fed females, with both groups producing peak MISO at 36 hpbf.

Figure S2 | The 20 kDa band present in ovary samples post-blood feeding when probed with the anti-MISO antibody is not a glycosylated or phosphorylated form of MISO. 10-15 blood-fed ovary samples were collected in protein extraction buffer, treated with (a) Deglycosylation Enzyme Cocktail (DeG enzyme, New England Biolabs) or (b) lambda-phosphatase (deP enzyme, New England Biolabs), and run on a gel. No band shifts were detected for the 20 kDa bands, indicating that they are not modified MISO protein.
Figure S3 | Knock-down efficiency of dsMISO in atria and ovaries. (a) Pools of 8-12 atria were collected from dsMISO and dsControl females 24 hours post-mating (hpm) and MISO expression relative to the housekeeping gene RPL19 was determined by qRT-PCR across 9 different experiments presented in Chapter 2. The MISO relative expression in dsMISO was then compared to this value in dsControl in a box and whisker plot. Top, middle, and bottom bars represent the 3rd quantile, median, and 1st quantile, respectively, with whiskers representing minimum and maximum values. The cross represents the mean. The mean percent knock-down efficiency was 88.6%. (b) Pools of 8-12 ovaries were collected from non-blood-fed females (0 hpbf) and at 3, 24, and 48 hpbf from dsMISO and dsControl mated females. MISO expression was significantly reduced in dsMISO ovaries relative to dsControl (Two-way ANOVA, dsRNA: F = 58.13, df = 1, p < 0.0001; time post-blood feeding: F = 5.34, df = 3, p < 0.01; dsRNA x time post-blood feeding: F = 4.47, df = 3, p < 0.05). Time post-BF = time post-blood feeding (c) Protein was extracted from 10-12 atria and ovaries from dsMISO and dsGFP females at various timepoints post-mating and post-blood feeding and probed for MISO levels by Western blotting. While MISO is depleted in the atria of dsMISO females, it does not seem to potently respond to knock-down in the ovaries. NBF = non-blood-fed
Figure S4 | MISO protein levels are increased in the atria and ovaries of mated females fed a *P. falciparum*-infected blood meal. (a) Ovaries and atria were collected 24 hpbf from females fed on a *P. falciparum* infected (+) or uninfected (-) blood meal and probed for MISO by Western blot. In 3 of 4 replicates, the level of MISO was increased in infected reproductive tissues relative to uninfected control. (b) The intensity of MISO bands was normalized to actin, then this value in infected tissues was normalized to the intensity in uninfected samples. The mean percent increase in band intensity was 15.7% when all 4 replicates were considered (n = 4, 95% confidence interval 0.488-1.825). Error bars represent standard errors of the means.
Table S1 | Transcriptional datasets inputted for correlation analysis

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tissues</th>
<th>Timepoints</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>20E-injected <em>An. gambiae</em> females</td>
<td>atria, spermatheca, ovaries, head</td>
<td>24 hpi</td>
<td>[1]</td>
</tr>
<tr>
<td>Blood fed <em>An. gambiae</em> females</td>
<td>whole bodies, ovaries, midgut, fatbody</td>
<td>3, 24, 72, 96 hpbf, 5 days pbf</td>
<td>[2]</td>
</tr>
<tr>
<td>Blood fed <em>An. gambiae</em> females</td>
<td>whole bodies</td>
<td>3 hpbf</td>
<td>[3]</td>
</tr>
<tr>
<td>Untreated <em>An. gambiae</em> females</td>
<td>head, midgut, salivary glands, rest of body</td>
<td>3 days old</td>
<td>[4]</td>
</tr>
<tr>
<td>dsEcR- injected <em>An. gambiae</em> females</td>
<td>atria, spermatheca, ovaries, head</td>
<td>24 hpm</td>
<td>[5]</td>
</tr>
<tr>
<td><em>An. gambiae s.l.</em> females, Tiassalé and Ngousso strains</td>
<td>malpighian tubules, midgut, abdomen</td>
<td>N/A</td>
<td>[6]</td>
</tr>
</tbody>
</table>

Transcriptional data from published and unpublished studies were included in the correlation network analysis performed in the Shiny R application. The sample type, tissue, and timepoints assayed in each experiment are outlined.
Table S2 | HIF promoter elements in Genomatix

<table>
<thead>
<tr>
<th>Matrix Family</th>
<th>Detailed Family Information</th>
<th>Matrix</th>
<th>Detailed Matrix Information</th>
<th>Genomic start position</th>
<th>Genomic end position</th>
<th>Distance from start codon</th>
<th>Chromosome</th>
<th>Strand</th>
<th>Core similarity</th>
<th>Optimized</th>
<th>Matrix similarity</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>V$HIFF</td>
<td>Hypoxia inducible factor, bHLH/PAS protein family</td>
<td>V$HRE.02</td>
<td>Hypoxia-response elements</td>
<td>24261161</td>
<td>24261177</td>
<td>618</td>
<td>2R</td>
<td>+</td>
<td>1</td>
<td>0.97</td>
<td>0.976</td>
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<tr>
<td>V$HASF</td>
<td>HIF-1 ancillary sequence family</td>
<td>V$HAS.01</td>
<td>HIF-1 ancillary sequence</td>
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<td>24261175</td>
<td>614</td>
<td>2R</td>
<td>-</td>
<td>1</td>
<td>0.91</td>
<td>0.937</td>
<td>tacCAGGtacc</td>
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<tr>
<td>V$AHRR</td>
<td>AHR-ant heterodimers and AHR related factors</td>
<td>V$AHRARNT.01</td>
<td>DRE (dioxin response elements), XRE (xenobiotic response elements) bound by AHR/ARNT heterodimers</td>
<td>24261233</td>
<td>24261257</td>
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<td>-</td>
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<td>DRE (dioxin response elements), XRE (xenobiotic response elements) bound by AHR/ARNT heterodimers</td>
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<td>V$HASF</td>
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<td>V$HIFF</td>
<td>Hypoxia inducible factor, bHLH/PAS protein family</td>
<td>V$HRE.02</td>
<td>Hypoxia-response elements</td>
<td>24261533</td>
<td>24261549</td>
<td>246</td>
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<td>V$HIFF</td>
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<td>V$ARNTL.01</td>
<td>Aryl hydrocarbon receptor nuclear translocator-like, homodimer</td>
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<td>0.87</td>
<td>0.89</td>
<td>0.891</td>
<td>gtaCggtGTTGatg</td>
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</table>

7 separate binding sites for hypoxia-related factors were found in the MISO promoter area as determined using Genomatix MatInspector software. Core similarity (matching to the most highly conserved 4 core nucleotides in a transcription factor binding site) cut-off was set to 0.75 (a minimum of 3 of 4 highly conserved nucleotides must be present). Matrix similarity (calculated by matching nucleotides in the promoter region to highly and less conserved regions) was set to an optimized value as determined by the software. Conserved nucleotides are highlighted in red. A more in depth description of this analysis can be found in the **Materials and Methods** section of Chapter 2.
### Table S3 | Primers used to assess gene expression by qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Primer direction</th>
<th>Primer sequence</th>
<th>Final primer concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MISO</td>
<td>AGAP002620</td>
<td>Forward</td>
<td>AGACGATGGAGGGACTGATG</td>
<td>300 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GGATTGCCTTTCTGTGCTG</td>
<td>300 nM</td>
</tr>
<tr>
<td>HIFα</td>
<td>AGAP002942</td>
<td>Forward</td>
<td>GGACGGAAGATATGAATAAG</td>
<td>300 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CAGATGCGTTAGATCATCC</td>
<td>300 nM</td>
</tr>
<tr>
<td>HIFβ</td>
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<td>Forward</td>
<td>CAAATGTTCCTCGTCATGTAC</td>
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<td></td>
<td></td>
<td>Reverse</td>
<td>TACACACCACGTACTCGATG</td>
<td>300 nM</td>
</tr>
<tr>
<td>Fatiga</td>
<td>AGAP003523</td>
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<td>GTATTCGTCTGGAGTGTTC</td>
<td>300 nM</td>
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<td></td>
<td></td>
<td>Reverse</td>
<td>CTATCCACGTATTTTGTC</td>
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</tr>
<tr>
<td>RPL19</td>
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<td>Forward</td>
<td>CCAACTCGCGACAAAACATTC</td>
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<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>ACCGGCTTCTTGTGATACAGA</td>
<td>900 nM</td>
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</table>

### SUPPLEMENTARY METHODS

**Protein deglycosylation assays**

To determine whether the 20 kDa band visible when ovarian samples are probed for MISO by Western is a glycosylated form of the protein, a modified protocol of the Protein Deglycosylation Mix (New England Biolabs) was utilized. The Deglycosylation Enzyme Cocktail in this mix can remove O- and N-linked glycans. 25 µg of protein from virgin and mated bloodfed ovary samples was denatured at 100°C for 10 minutes in 1X Glycoprotein Denaturing Buffer, chilled on ice, then incubated in 1X GlycoBuffer 2, 1% NP-40, and 5 µl Deglycosylation Enzyme Cocktail (sufficient to deglycosylate 100 µg glycoprotein) for 15 hours at 37°C. For each sample, a control was run containing all the reaction components except for the Deglycosylation Enzyme Cocktail, and a Fetuin control (contains both O- and N-linked glycans) was included. Following incubation with the Cocktail, samples were analyzed by Western blot assay probing for MISO as described in the **Western blotting** Methods section in Chapter 2.
Protein dephosphorylation assays

To determine whether the 20 kDa band visible when ovarian samples are probed for MISO by Western is a phosphorylated form of the protein, 25 µg of protein from pools of 12 ovaries collected at 6, 24, and 36 hpbf, as well as from ovaries collected after oviposition were treated with 400 units lambda phosphatase (New England Biolabs) in a reaction mixture containing 1X NEB Buffer for PMP and 1 mM MnCl₂ for 30 minutes at 30˚C. For each sample, a control was run containing all the reaction components but with the lambda phosphatase replaced with glycerol. Following incubation at 30˚C, the samples were analyzed by Western blot assay probing for MISO as described in the Western blotting Methods section in Chapter 2.

REFERENCES


APPENDIX B:
Investigating the interaction between JNK signaling and MISO

Overview

This supplementary chapter outlines additional experiments performed to elucidate whether MISO acts within c-Jun N-terminal kinase (JNK) signaling to mediate tolerance to \textit{Plasmodium falciparum} infection. The JNK pathway is activated when a mitogen-activated protein kinase (MAPK) kinase phosphorylates JNK, which in turn phosphorylates the AP-1 transcription factor heterodimer composed of Fos and Jun \(^1\). This transcription factor heterodimer can then mediate the stimulation of the transcription of effector genes.

\textbf{MISO is regulated by the AP-1 transcription factor Fos}

To investigate other signaling pathways that may be regulating MISO, we used Genomatix MatInspector software \(^2,3\) to look for putative transcription factor binding sites in \textit{MISO}'s promoter region. This analysis returned a number of binding sites with high core and matrix similarity for transcription factors involved in embryonic development and morphogenesis, including homeobox/homeodomain transcription factors, adding support to our results from transcriptional and protein analysis of MISO in \textit{Anopheles gambiae} ovaries. This analysis also identified a number of putative binding sites for AP1-related transcription factors, which we decided to investigate further because (1) we have evidence from RNAseq analysis performed on female \textit{An. gambiae} tissues post-mating that the \textit{An. gambiae} c-Jun N-terminal kinase (JNK) gene \textit{AGAP009460} is highly upregulated in mated atria relative to virgins at 3 hpm, preceding the peak of \textit{MISO} expression at 24 hpm; (2) JNK signaling has been implicated in the anti-plasmodial response of mosquitoes infected with \textit{Plasmodium berghei} \(^4\) and \textit{P. falciparum} \(^5\); and (3) JNK and other programmed cell death signaling pathways are required for \textit{Drosophila} ovarian (reviewed in \(^6\)) and embryonic (reviewed in \(^7\)) development, often in relation to 20E-mediated signaling.
We silenced the AP-1 transcription factor Fos (AGAP001093) using dsRNA targeting this gene in one day-old females, and 3 days post-injection we collected ovaries of non-blood-fed females. At the same time, we blood fed these dsFos (and control dsGFP) females, collected ovaries 36 hours post-blood feeding (hpbf), then analyzed the level of MISO by Western blot. We found that females silenced for Fos produced greater amounts of MISO protein in their ovaries post-blood feeding (Figure S5a), with a mean increase of 74.1% in dsFos blood-fed ovaries relative to dsControl (n = 3, 95% confidence interval 0.36-3.13) (Figure S5b).

Conversely, MISO did not affect the expression of AP-1 transcription factors following blood feeding. As there are no commercially available antibodies optimized to detect Jun or Fos in Anopheles or Drosophila, we utilized a transcriptional approach and found that although the levels of Fos (Figure S5c) and Jun (AGAP006386) (Figure S5d) increased in the ovaries of blood-fed females over time (Two-way ANOVA, Fos: F = 19.96, df = 3, p < 0.0001; Jun: F = 13.77, df = 3, p < 0.001), expression was not impacted by MISO silencing (Two-way ANOVA, Fos: F = 0.0155, df = 1, p > 0.05; Jun: F = 0.096, df = 1, p > 0.05) (Figure S5c and S5d). Moreover, we were unable to determine whether MISO or P. falciparum infection impact JNK signaling, as across multiple replicates of Western blots on reproductive tissues of dsMISO and dsGFP females fed on uninfected or P. falciparum-infected blood, the signal for the active phosphorylated-JNK (pJNK) was consistently below detection limits (data not shown). These results suggest that MISO expression is downstream of JNK signaling and in part regulated by AP-1 transcription factors, but that these factors may act more to repress or downregulate its expression rather than promote it.
Figure S5 | MISO produced following blood feeding is increased in dsFos females, but transcription of AP-1 transcription factors are not affected by dsMISO. (a) 10-12 ovaries were collected from dsGFP and dsFos females 36 hpbf and probed for MISO levels by Western blotting. (b) Across three replicates, MISO levels are increased in dsFos females relative to controls, with a mean induction of 74%. Error bars represent standard errors of the means. (c) qRT-PCR analysis of Fos expression shows that there is a significant induction of this gene post-blood feeding (F = 19.96, df = 3, p < 0.0001), but no effect of MISO knock-down (F = 0.0155, df = 1, p > 0.05), or of the interaction of dsRNA treatment with time-post bloodfeeding (F = 0.135, df = 3, p > 0.05). (d) qRT-PCR analysis of Jun expression shows that there is a significant induction of this gene post-blood feeding (F = 13.77, df = 3, p < 0.001), but no effect of MISO knock-down (F = 0.096, df = 1, p > 0.05), or of the interaction of dsRNA treatment with time-post bloodfeeding (F = 0.331, df = 3, p > 0.05). Results of the qRT-PCR are from 3 replicates, with error bars representing standard errors of the means.
Concluding remarks

While we determined that MISO levels in the ovaries are modulated by the AP-1 transcription factor Fos, we were unable to establish a decisive link between JNK signaling and *P. falciparum* infection or MISO silencing, as phosphorylated JNK was consistently below levels of detection in the reproductive tissues of *An. gambiae* females post-blood feeding. Combined with the observation that MISO does not appear to affect apoptosis in the ovaries (Chapter 2), these results suggest that MISO may function downstream of AP-1 signaling, but that its role within this pathway is not to regulate apoptosis in the ovarian follicles. The results presented in Chapter 2 suggest MISO functions within hypoxia-induced signaling, and indeed, studies suggest HIF and AP-1 signaling may function in parallel to induce gene expression in hypoxic conditions. *Fos* and *Jun* transcription were shown to increase in hypoxic cell lines (reviewed in 8), and both AP-1 and HIF transcription factors were demonstrated to function together to increase production of the pro-growth vascular endothelial growth factor (VEGF) 9. As a result, MISO may act downstream of both pathways to potentiate hypoxia-induced signaling, potentially in a role to promote cellular growth, though further studies examining the effects of impairing HIF processes on MISO and JNK signaling will be necessary.

SUPPLEMENTARY METHODS

RNA interference

To knock-down gene expression of *Fos* (*AGAP001093*), a 357 bp region of the coding sequence was cloned into pCR2.1 (Thermo Fisher Scientific). The same plasmid was used for the dsGFP control as described in Chapter 2. Using primers containing the T7 sequence and specific to the plasmid backbone, PCR reactions were performed on 250-1000 pg plasmid DNA to amplify the coding sequence and generate knock-down constructs specific to each gene. The primers used to amplify the *Fos* cassette were Forward: 5’-TAATACGACTCACTATAGGGCCGCCAGTGTGCTGGAA-3’ and Reverse: 5’-
dsRNA was then generated and knock-down studies were performed on virgin 1 day old females as described in the RNA interference section in Chapter 2.

**Western blotting**

Assays were performed as described in the Material and Methods of Chapter 2 with a 1:1000 dilution of anti-MISO polyclonal antibody (Rabbit). Attempts to detect pJNK were done using 1:1000 dilutions of phospho-SAPK/JNK antibody (Cell Signaling Technology, #4668S) using the Western blotting methods detailed in Chapter 2. Quantification of band intensity was performed using the LI-COR software Image Studio™ Lite by normalizing MISO levels to actin, and then normalizing those values in dsFos females to dsGFP females.

**Gene expression analysis in mosquito tissues by quantitative RT-PCR**

Experiments were performed as described in this same section in the Materials and Methods of Chapter 2 on the 3 replicates of ovaries collected at 0, 3, 24, and 48 hpbf in dsGFP and dsMISO females. The qRT-PCR primers used were the RPL19 primers described in Chapter 2, as well as:

**Fos:** Forward 5'-CAATTACCTTACTGCGGTAGC-3'
Reverse 5'-GTTGTGACTCTTCATATTGGCG-3'

**Jun:** Forward 5'-AGGGCAAGTTTTGAATGCAC-3'
Reverse 5'-CACGCACCTTCTCCCTTTGT-3' (from 5)

**REFERENCES**


Figure S6 | Eggs laid by females in crosses between wAnga-infected and uninfected mosquitoes. The number of eggs laid by each female was determined by counting eggs laid on filter papers in each oviposition cup 4 days after egg laying (Kruskal-Wallis, $\chi^2 = 0.686$, d.f. = 3, $p > 0.05$). wAnga-infected (green) and uninfected (grey) females (right) and males (left) were identified by 16S nested PCR post-hoc, and mating couples were divided into 4 groups (wAnga-negative couples, dark grey; wAnga-positive couples, purple; wAnga-positive females mated to wAnga-negative males, red; wAnga-negative females mated to wAnga-positive males, blue).
Figure S7 | Alignment of wAnga 16S rDNA sequences. 221 blood-fed *Anopheles coluzzii* females were collected from the walls of houses in the Vallée du Kou, females were dissected five days later, and the DNA of thoraxes and abdomens was extracted. Using *Wolbachia*-specific primers, the 16S rDNA region of wAnga was amplified by nested PCR. The sequence of all PCR-positive samples was determined using Sanger sequencing of the 412 bp product, and all sequencing results were aligned to wAlbB (Accession number CAGB01000162) and wPip (Accession number EU096232) reference 16S rDNA sequences (Silva Database). An alignment of a subset of 20 randomly selected sequences from wAnga-infected females was constructed using the CLC Sequence Viewer software (CLC Bio, Qiagen). Nucleotides are numbered according to the wAlbB 16S rDNA sequence, with the aligned region representing bases 1215-1334.
Table S4 | wAnga and *Plasmodium* prevalence in natural *An. coluzzii* populations in 2014. Samples from 221 blood-fed females collected from houses in VK5 and allowed to develop eggs and oviposit were analyzed for the presence of wAnga and *Plasmodium* by 16S nested PCR and 18S qPCR, respectively. The table shows the number of infected females identified for each infection and the percentage of the total. The proportional data is graphed in Figure 3.4a.

<table>
<thead>
<tr>
<th></th>
<th>wAnga +</th>
<th>wAnga –</th>
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<tbody>
<tr>
<td><em>Plasmodium +</em></td>
<td>1 (0.5%)</td>
<td>11 (5.0%)</td>
</tr>
<tr>
<td><em>Plasmodium –</em></td>
<td>115 (52.0%)</td>
<td>94 (42.5%)</td>
</tr>
</tbody>
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