Journal Pre-proof

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Corrado Minetti, Victoria A. Ingham, Hilary Ranson

PII: S2214-5745(20)30008-0

DOI: https://doi.org/10.1016/j.cois.2019.12.001

Reference: COIS 653

To appear in: *Current Opinion in Insect Science*

Please cite this article as: Minetti C, Ingham VA, Ranson H, Effects of insecticide resistance and exposure on *Plasmodium* development in *Anopheles* mosquitoes, *Current Opinion in Insect Science* (2020), doi: https://doi.org/10.1016/j.cois.2019.12.001

Effects of insecticide resistance and exposure on *Plasmodium* development in *Anopheles* mosquitoes

Short title: Impact of insecticides on malaria development

Corrado Minetti[1], Victoria A. Ingham[1], Hilary Ranson[1]

[1]Department of Vector Biology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L35QA, United Kingdom

Highlights

- Changes in *Anopheles* vector competence for *Plasmodium* parasites have been linked to insecticide resistance status
- Insecticide exposure is detrimental for *Plasmodium* in the midgut lumen
- Xenobiotic detoxification reactions in mosquito tissues can affect *Plasmodium* development
- It is likely that other resistance mechanisms impact parasite development directly or indirectly.
- The lack of research on this topic is surprising, given the critical impact that any interactions may have on the epidemiology or malaria in an era of widespread pyrethroid resistance.

Abstract

The spread of insecticide resistance in anopheline mosquitoes is a serious threat to the success of malaria control and prospects of elimination, but the potential impact(s) of insecticide resistance or sublethal insecticide exposure on *Plasmodium-Anopheles* interactions are poorly understood. Only a few studies have attempted to investigate such interactions, despite their clear epidemiological significance for malaria transmission. This short review provides an update on our understanding of the interactions between insecticide resistance and exposure and *Plasmodium* development, focusing on the mechanisms which might underpin any interactions, and identifying some key knowledge gaps.

Keywords: Anopheles; Plasmodium; insecticide resistance; Interaction

Introduction

Progress in reducing the malaria burden has recently stalled: globally the estimated number of malaria cases per 1000 population at risk stood at 59 for three consecutive years (2015-2017), and some high burden African countries even experienced an increase in cases [1]. Insecticide resistance (IR) to all the major insecticides in anopheline mosquitoes constitutes a serious threat to the

achievements of malaria control and the prospects of elimination. Between 2010-2017, 85% of countries with available data reported resistance to at least one insecticide class and particularly resistance to pyrethroids [1], which is now widespread in the major African vectors [2]. Pyrethroid resistance is particularly concerning since this insecticide class is used in all insecticide treated nets (ITNs) currently on the market and the scale up in ITN use is the mainstay of most African malaria control programmes.

In mosquitoes IR is largely attributed to i) changes to the insecticide target site, ii) increased insecticide metabolic detoxification through increased action of proteins and iii) modifications in the thickness and chemical composition of the cuticle. Recently, other proteins have been implicated in IR expression and regulation in *Anopheles gambiae*, the major African malaria vector [3••,4,5]. In the field, mosquitoes can be exposed to sublethal doses of insecticide through ITNs or indoor residual spraying (IRS), either due to insecticide decay, resulting in surface concentrations below target doses, or the presence of resistant mosquitoes that are not killed by the target dose. The potential impact(s) of IR and sublethal insecticide exposure on malaria vector competence, defined as the mosquito's capacity to support the development of *Plasmodium* parasites up to the infective sporozoite stage, is still poorly understood. In the mosquito *Plasmodium* takes an average of two weeks to develop and during this development, is exposed to multiple mosquito tissues hence physiological changes elicited by IR or exposure could affect the likelihood of development of the final infectious stage, the sporozoite [6]. A potential link between IR and vector competence has been observed in other mosquito-borne pathogens. At 12 days post infection with the filarial parasite *Wuchereria bancrofti*, none of the laboratory-selected organophosphate resistant *Culex quinquefasciatus* harboured the parasite L3 infective stage whereas 76% of susceptible insects were infected [7]. Following infection with West Nile virus, resistant *Cu. quinquefasciatus* carrying the *ester*^{2} and *ace-1* IR mutations showed a significantly higher viral dissemination rate compared to susceptible insects [8••].

In this review we evaluate the available evidence of the effect(s) of physiological IR and exposure on within-vector *Plasmodium* development, the potential mechanisms and future research directions.

Insecticide resistance mechanisms in African malaria vectors

All the major IR mechanisms have been reported in African malaria vectors (Table 1), often occurring in combination, causing cross-resistance to different insecticide classes and differing between populations across the geographical range of a given species [3••].

What is the evidence that insecticide resistance status affects *Plasmodium* development?

Only a few studies have explored the impact of IR on *Plasmodium* development (Table 2). Results show contrasting effects on either parasite prevalence (i.e the proportion of exposed mosquitoes infected) or intensity (i.e the number of parasite oocysts or sporozoites in infected mosquitoes). The presence of the target-site mutations *kdr* or *ace-1* in homozygosis in isogenic lines were associated with an increased parasite prevalence but a reduced intensity in *An. gambiae* [26••], whereas an increase in both was observed feeding *Plasmodium* to *kdr*-homozygous mosquitoes reared from larvae collected in nature [27]. On the contrary the L119F-GSTe2 mutation, associated with pyrethroid metabolic resistance via more efficient GST activity [18], was linked in homozygosity to a reduced oocyst prevalence but to a marginally (but non-significantly) higher intensity in lab-infected

An. funestus [28]. Additionally, L119F-GSTe2 was associated with higher sporozoite prevalence in field-collected indoor-resting homozygous mosquitoes [29].

Whilst experiments on wild caught field mosquitoes could be argued to be most informative, it is important to note that under insecticide pressure, resistant mosquitoes in the field have a higher chance to survive the parasite extrinsic incubation period (EIP) (i.e. the time taken by *Plasmodium* to develop in the vector and become transmissible), and this, rather than any specific relationship between IR and parasite development, may explain the link between IR and presence of parasites in the field. Hence studies should first focus on the ability of IR and susceptible mosquito populations to develop salivary gland infections via feeding on infectious gametocytes (preferably obtained from sympatric human populations) [27]. It is also important to study naturally selected resistant populations that may contain multiple resistant mechanisms as this is the norm, rather than exception, in African malaria vectors.

What is the evidence that sublethal insecticide exposure affects *Plasmodium* development?

Studies on *P. falciparum* (Table 3) consistently showed a reduction in oocyst prevalence and intensity following sublethal exposure to pyrethroids and other insecticides, regardless of the resistance status of the insects. The effect was observed only when exposure occurred around the time of the infectious blood meal (N Hill, 2002) [31], suggesting that insecticides interfere with the early midgut stages of the parasite. Interestingly, it has also been shown that the early midgut development of *P. falciparum* is arrested by exposing *An. gambiae* to surfaces treated with the antimalarial drug atovaquone [32••]. Whether this phenotype is caused by direct contact between the parasite and the xenobiotic (or its toxic by-products) reaching the midgut, or to the effects of the induced detoxification response is unclear. None of the studies on insecticide exposure took into consideration the sporozoite stage.

Putative mechanisms

In the mosquito *Plasmodium* parasites face many physical, chemical, immune and microbial barriers [34, 35, 36, 37•,38], in different tissues and at different times (Figure 1). Many of these barriers may be impacted by the IR status, and/or insecticide exposure, as discussed below.

Overexpression of detoxification enzymes in resistant mosquitoes, or induction of the same enzymes after insecticidal exposure, may either inhibit or enhance parasite development via regulation of the amount of cytotoxic reactive oxygen and nitrogen species (ROS, RNS) [6]. For example while cytochrome P450s (CYPs) increase the levels of ROS during their activity, GSTs act in the opposite way reducing tissue oxidative stress [39]. Whether changes in the redox potential affect *Plasmodium* may depend on whether the expression of these enzymes is localized in specific tissues. Studies so far have shown that in *An. gambiae* the activity of CYPs is high in both oenocytes (fat body) and midgut [40], and overexpression of certain CYPs have been reported in the midgut of pyrethroid-resistant *An. gambiae* [41]. Furthermore chemical synergists such as piperonyl butoxide (PBO) [42], which inhibits the activity of detoxification enzymes and that can be added to pyrethroid-treated surfaces to combat metabolic resistance, may affect how these mechanisms impact on *Plasmodium*. Changes in the midgut redox conditions may explain the reported detrimental effects of metabolic resistance or exposure to xenobiotics and insecticides on the midgut stages of filarial [7] and malaria [28,32,33] parasites.

Since ROS/RNS also act as both effectors and mediators in *Anopheles* anti-plasmodial immunity systemically and specifically in the midgut lumen and epithelium [35], changes in their amount may have pleiotropic effects on other immune responses. Potential links between IR, detoxification and immune responses in *Anopheles* mosquitoes have only been suggested indirectly. It has been shown in various insect species that sublethal exposure to pesticides can affect the humoral and cellular components of the immune system and their ability to fight infection [43•].

Transcriptomic studies comparing genetically unrelated pyrethroid-resistant and susceptible mosquitoes reported a constitutive overexpression of immunity-related genes: antimicrobial peptides (AMPs) (defensin, cecropin) in resistant *An. gambiae* [44] and nitric oxide synthase (NOS) in resistant *An. stephensi* [45]. AMPs and nitric oxide have important cytotoxic anti-plasmodial activity [35]. In another study, isogenic *Cu. pipiens* mosquitoes carrying the *ester*^{4} gene duplication (responsible for metabolic resistance due to carboxylesterase overproduction), but not those with the *ace-1* mutation, showed a higher constitutive expression of the AMPs gambicin and defensin, transferrin (a regulator of iron metabolism associated to innate immunity) and nitric oxide synthase genes compared to the wild type [46••]. Resistant *Cu. pipiens* also exhibited a significantly higher activity of the enzyme phenoloxidase (a key enzyme in the mosquito melanisation cascade) [47]. However, in both studies none of the differences observed in lab-selected mosquitoes were found in field-caught resistant insects [46••,47]. Changes in the expression levels of salivary glands-specific proteins have also been reported in insecticide-resistant *An. gambiae* [4,48], but whether such alterations affect *Plasmodium* competence and the sporozoites invasion process, viability or infectivity is unknown.

The presence of a cross-talk between detoxification and infection responses is suggested by a study in which *An. gambiae* mosquitoes were infected with the rodent parasite *Plasmodium berghei* and the expression of detoxification enzymes was measured at different timepoints [49]. Various alterations were observed in concomitance of ookinete midgut invasion and sporozoite release in the hemocoel, and most notably the overexpression of CYP6M2 [50] (implicated in pyrethroid resistance) in both the mosquito midgut and fat body [49]. In this study the effect of infection on phenotypic IR was not evaluated using bioassays. However, when *kdr*-homozygous *An. gambiae* were infected with *P. falciparum* and then exposed to DDT at one, seven or 14 days post-infection, infected mosquitoes showed a significantly higher mortality than uninfected ones at the first two timepoints [51]. In another study, *kdr* and *ace-1* homozygous *An. gambiae* infected with *P. falciparum* showed a reduced survival compared to infected susceptible mosquitoes [52], with the authors proposing resistance mutations exerting a cost on mounting an anti-plasmodial response by interfering directly or indirectly with immune system factors.

Less clear is how pathogens can be affected by insecticide target-site mutations [8••,26••,27,28]. Isogenic lines in which IR alleles are introgressed into a susceptible background enable the impact of individual resistance mechanisms to be evaluated [8••,26••,46••,53], but genes which are involved in immunity and vector competence and in linkage disequilibrium with IR alleles can also be introgressed in such crosses. For example, the voltage-gated sodium channel (VGSC) gene on which the *kdr* mutation occurs is part of an haplotype including also a gene coding for a serine protease (*ClipC9*) potentially linked to anti-plasmodial immunity [53]: indeed the number of *P. falciparum* oocysts in *An. coluzzii* significantly increased after *ClipC9* silencing by RNA interference, whereas silencing the VGSC gene did not alter infection susceptibility. This mechanism could partially explain the putative association between target-site resistance and increased *P. falciparum* susceptibility reported in both laboratory-generated [26] and wild mosquitoes [27].

Midgut microbiota also influence malaria competence via immune system stimulation, production of toxic metabolites or competition for energetic resources, and potentially via the synthesis of the peritrophic membrane [37•]. Microbiota-mediated effects on *Plasmodium* are likely not confined to the midgut lumen [54], and there is some suggestive evidence of a relationship between insecticide resistance and microbiome composition in mosquitoes [55]. Whether IR or exposure affect microbiome composition and function and the interactions with malaria parasites warrants further investigation.

Plasmodium actively scavenges mosquito nutritional resources such as lipids, which play an essential role in the sporozoites maturation and infectivity [56,57••]. Expression of IR has been associated with reduced energetic reserves in *C. pipiens* [58] and, if IR exerts a similar affect in *Anopheles*, this may act as a constraint for *Plasmodium* development. Mosquito reproduction is also intimately linked to *Plasmodium* development. Disruption of the signalling pathways of the steroid hormone 20-hydroxyecdysone (20E), which regulates many of the processes of the mosquito gonotrophic cycle, significantly affects *P. falciparum* oocyst and sporozoite development in *An. gambiae* [57••, 59]. One of the currently proposed strategies for controlling resistant vector relies on using insect growth regulators (IGRs) in ITNs, like the juvenile hormone (JH) analog pyriproxyfen (PPF) [60]. It cannot be excluded that IR or exposure to insecticides or IGRs may impact *Plasmodium* by interfering with the vector hormonal signalling pathways.

Potential effects of IR/exposure should be studied beyond their impact on individual *Plasmodium* developmental stages and their numbers. None of the studies so far have examined whether the length of the parasite extrinsic incubation period (EIP) was affected. The EIP is a crucial parameter for malaria transmission and it is influenced by a variety of biotic and abiotic factors [61•].

Although not the focus of this review, IR/exposure effects on mosquito longevity [6] and behaviour [62] must also be taken into account given their fundamental contribute to vectorial capacity and malaria transmission.

Conclusions and perspectives

Extensive further research is needed to understand in which ways IR/exposure affect within-vector *Plasmodium* development. Studies so far have largely relied on isogenic mosquito lines fed laboratory-grown parasites, which do not necessarily reflect the field situation where insecticide resistance is generally polygenic. Furthermore, the lack of sympatric field-colonized susceptible and resistant mosquito populations makes disentangling the infection phenotype contribution of IR alleles/mechanisms compared to immunity/vector competence genes difficult, while indirect approaches looking at correlations between parasite infection and IR alleles in the field are confounded by the effect of the latter on mosquito longevity in areas where insecticides are being used. Dissecting the molecular pathways of the potential cross-talk between detoxification mechanisms and mosquito immunity, energetics and reproduction and their cascade effects on all aspects of *Plasmodium-Anopheles* interactions should be considered a priority. This aspect is particularly relevant for malaria control and prospects of elimination when novel chemistries (including synergists or IGRs on ITNs) for vector control are introduced in field settings, rising the urgent need to test these chemistries to assess any unintended effects on malaria transmission. In addition to delineating the mechanisms of interaction between pyrethroids and parasite development, it is critical that vector control tools containing novel chemistries (including synergists or IGRs on ITNs) are assessed for any unintended effects on malaria transmission that may be caused by interactions with parasite development.

Conflict of interest: none

Acknowledgements

The authors would like to thank M. Bernardi for graphic assistance. V.I is supported by an MRC Skills Development Fellowship (MR/R024839/1) and H.R is supported by a GCRF award, the Partnership for Increasing the Impact of Vector Control (PIIVeC) is funded by the Medical Research Council of the UK (grant number MR/P027873/1) through the Global Challenges Research Fund.

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Figure Legend

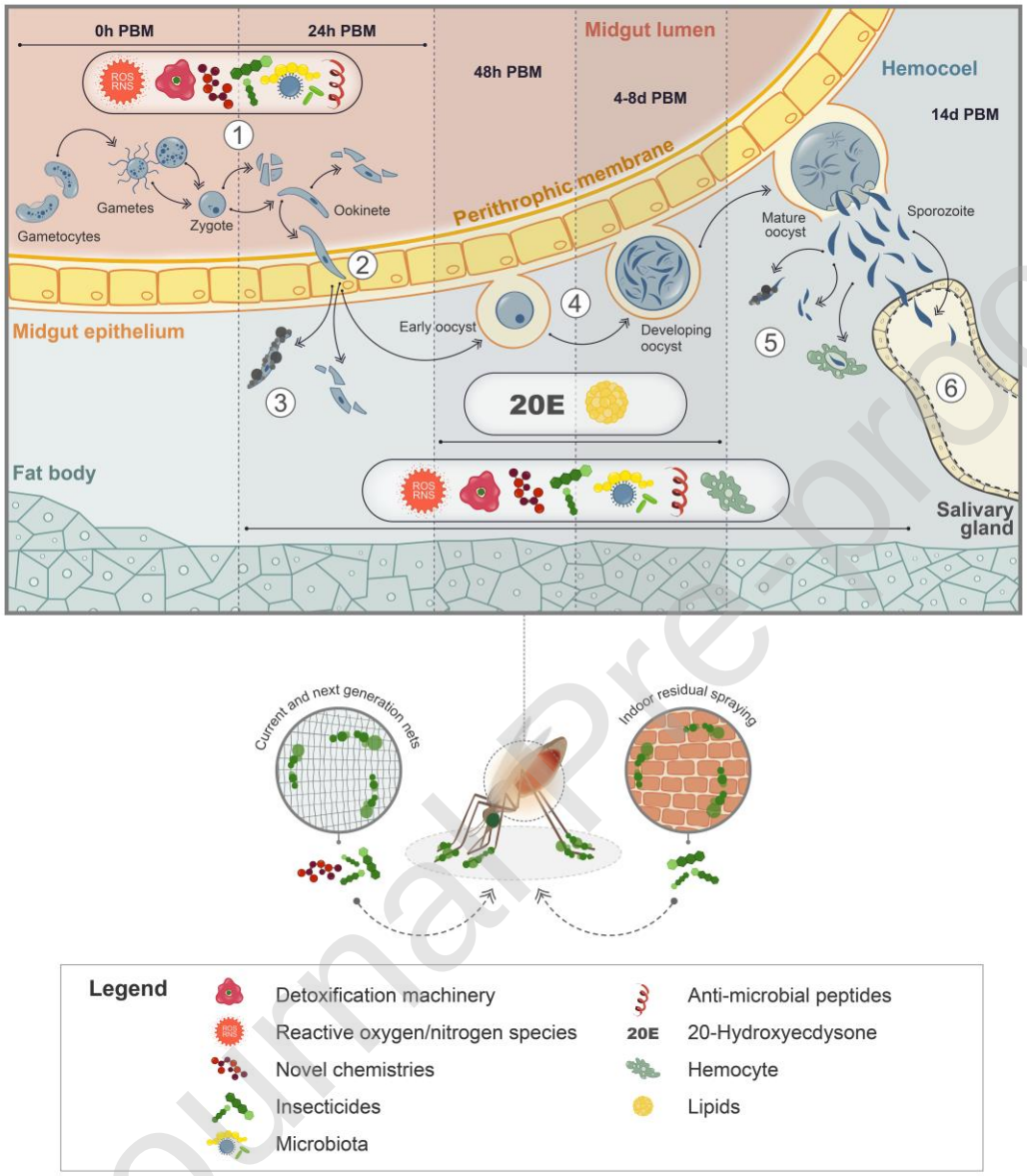

Figure 1: Anti-plasmodial barriers in *Anopheles* and ways they may be affected by insecticide resistance (IR) or sublethal exposure to insecticides and novel chemistries for vector control. In the midgut lumen (1) *Plasmodium* sexual stages and ookinetes may be affected by i) direct contact with vector control compounds or their by-products after metabolization, i) changes in the redox potential (ROS/RNS amounts) due to overexpression of detoxification enzymes/nitric oxide synthase (NOS) (in IR) or as a result of their activity (exposure), ii) changes in the amounts and activity of antimicrobial peptides (AMPs) (in IR), or iii) changes in the microbiome composition and activity (IR/exposure). All these mechanisms could play a role in multiple tissues affecting the parasite even past the midgut barrier (3-5) and up to the sporozoite stage in salivary glands (6). Other more localized processes may be modified by IR/exposure. During the penetration of the

midgut epithelium (2) the anti-ookinete defences based on ROS/RNS production and associated pathways, leading to either parasite melanisation or lysis (3), may be altered by IR via overexpression of NOS or other enzymes (for example of the melanisation cascade). Oocyst development (4) may be affected by IR/exposure interfering with i) lipids trafficking and hormone signalling (for example involving 20-hydroxyecdysone) or ii) the differentiation, numbers and activity of the hemocytes, the latter being regulated through ROS signalling and playing a role in AMPs production and pathogen phagocytosis or melanisation which may affect the parasite sporozoite stage (5) after its release in the hemocoel. Ultimately, the capacity of sporozoites to invade the salivary glands (6) and their survival may be affected by overexpression of uncharacterized proteins associated with an IR genotype or phenotype. Perturbations in all the above mechanism, either overall or in a tissue-specific manner, may affect the average 14 days duration of *Plasmodium* extrinsic incubation period. PBM, post blood meal.

Table 1: Insecticide resistance mechanisms and their molecular determinants identified in African malaria vectors. The term *An. gambiae* is used to indicate both *An. gambiae* s.s and *An. coluzzii*.

Type of resistance	**Major mechanism(s) involved**	**Insecticide class(es)**	**Molecular determinant(s) and *Anopheles* species**
Target-site	Reduced insecticide toxicity through point mutations leading to structural modifications of target proteins	PYR	***kdr*** - *An. gambiae*, *An. arabiensis* [9]
		OP, CA	***ace-1*** - *An. gambiae* [9]*; An. funestus* [10]
		OC	***kdr*** - *An. gambiae* [11] ***Rdl*** - *An. gambiae*, *An. arabiensis* [12]; *An funestus* [13]
Metabolic	Increased insecticide detoxification through increased metabolism and clearance	PYR	**CYPs** - *An. gambiae* [14]; *An. arabiensis* [15]; *An. funestus* [16] **GSTs** - *An. gambiae* [17]; *An. funestus* [18] **CCEs –** *An. gambiae* [19] **ABC-transporters** - *An. gambiae* [20]
		OP	**CYPs** - *An. gambiae* [14] **CCEs** - *An. arabiensis* [21]
		CA	**CYPs** - *An. funestus* [10] **CCEs** - *An. gambiae* [22]
		OC	**CYPs** - *An. gambiae* [14] **GSTs –** *An. gambiae* [23]; *An. funestus* [18]
Cuticular	Reduced insecticide penetration via cuticle thickening or altered composition	PYR	**CYPs** - *An. gambiae* [24]; *An. funestus* mechanism unknown [25]
Sequestration/ direct binding	Direct binding of insecticide, most likely leading to slow release for subsequent metabolic clearance	PYR	**α-crystallins and hexamerins with putative insecticide binding function** - *An. gambiae* [3••]
		OP, CA	**Salivary proteins D7(r2, r4) with putative insecticide binding function** - *An. gambiae* [4]

PYR= pyrethroids; OP= organophosphates; CA= carbamates; OC= organochlorines; *kdr*= knockdown resistance mutations in the voltage-gated sodium channel (VGSC); *ace-1*= mutation in the acetylcholinesterase enzyme; *Rdl*= mutation in the gamma-aminobutyric acid (GABA) receptor; CYP= cytochrome P450; GST= glutathione S-transferase; CCE= carboxylesterase; ABC= ATP-binding-cassette

Table 2: Studies comparing *Plasmodium* development in insecticide resistant and susceptible mosquitoes. Only studies using natural vector-parasite combinations were included.

Vector – parasite combination	Insecticide resistance mechanism	Effect(s) on *Plasmodium* in resistant mosquitoes	Reference
An. gambiae (Iso) – *P. falciparum**	*ace-1*, *kdr*	↑ooc/spo prevalence (*ace-1*, *kdr*), ↓ooc/spo intensity (*kdr* only)	[26••]
An. gambiae, *An. coluzzii* (w) – *P. falciparum**	*kdr*~	↑ooc/spo prevalence/intensity	[27]
An. gambiae (w) – *P. falciparum***	*kdr*~	↑spo prevalence	[30]
An. funestus (w) – *P. falciparum**	L119F-GSTe2~	↓ooc (homozygous only) (spo nd)	[28]
An. funestus (w) – *P. falciparum***	L119F-GSTe2~	↑spo prevalence	[29]

Iso= isogenic laboratory line; w= reared from field-collected larvae; *ester*^{2,4}= esterase A2/B2 and A4/B4 gene duplication (overproduction of esterases); *ace-1*= mutation in the acetylcholinesterase enzyme; *kdr*= knockdown resistance mutations in the voltage-gated sodium channel (VGSC); GST= glutathione-S-transferase; ooc= oocyst stage; spo= sporozoite stage; nd= not determined; *laboratory infection; **natural infection determined in field-collected adult mosquitoes; ~resistance status determined by post-dissection genotyping

Table 3: Studies evaluating the effects of insecticide sublethal exposure on *P. falciparum* development. Only studies using natural vector-parasite combinations were included.

Mosquito species and resistance status	Insecticide exposure details	Effect(s) on parasite development	Reference
An. stephensi (l – sus)	0.25% permethrin for 30 min, immediately pi	↓ooc prevalence	(N Hill, PhD thesis, London School of Hygiene and Tropical Medicine, 2002)
An. gambiae (l – res *ace-1* and *kdr*)	0.1% bendiocarb (*ace-1*)/4% DDT (*kdr*) for 1h, 18 hr bi	↓ooc prevalence (both strains)/ intensity (*ace-1*)	[31]
An. gambiae (w – res *kdr*)	deltamethrin (2.5-5 mg/m^2 vs 10-16.7 mg/m^2 doses) for 5 min, ≤3 hr pi	↓ooc prevalence/ intensity	[33]

l=laboratory line; w=reared from field-collected larvae; sus=insecticide susceptible; res= insecticide resistant; pi=post infectious bloodmeal; bi=before infectious bloodmeal; hr=hours; min=minutes; ooc= oocyst stage