Effectiveness of Pyriproxyfen and Olyset Duo in Controlling Insecticide Resistant Mosquito Populations in Burkina Faso

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by

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Abstract

The escalation of Long-Lasting Insecticidal Nets (LLINs) mass distribution marked the beginning of a period of malaria decline in sub-Saharan Africa. However, the emergence and spread of insecticide resistance in malaria mosquitoes is a threat to the effectiveness and sustainability of this vector control method. In this context, it is necessary to design and evaluate new compounds and methods that attenuate or even reverse the insecticide resistance trend.

Olyset Duo is a novel LLIN that combines the insecticide properties of permethrin and the chemosterilising effect of pyriproxyfen (PPF). The rationale is that resistant mosquitoes that survive the contact with the net would not be able to transfer the resistance genes to the offspring, eventually influencing the resistant phenotype of the mosquito population.

In the first part of this study the sterilising and sub-lethal effect of PPF and Olyset Duo was evaluated by a range of bioassays with laboratory and wild mosquito populations. PPF significantly affected the longevity, oogenesis, oviposition and hatching rate of susceptible and resistant mosquitoes, although the effect was partially diminished on the resistant colonies. The possible reasons and implications of PPF performance under controlled conditions are discussed.

The second part of this thesis was done within the context of an Olyset Duo Randomised Controlled Trial (RCT) set in Banfora District, Burkina Faso. The RCT had a stepped-wedge design which ensured that Olyset Duo nets gradually replaced Olyset nets in randomly allocated cluster of villages until the Olyset Duo coverage was total. Wild mosquitoes collected in sentinel sites with Olyset Duo showed evident signs of reproductive impairment even after 1 year of deployment. Insecticide resistance strength was monitored during the RCT in several sentinel sites, and time-response data showed an overall reduction of permethrin resistance strength after the distribution of Olyset Duo.

This is the most detailed study on the effect of Olyset Duo on key entomological factors of wild mosquito populations. The standardised protocols as well as the dataset obtained are valuable information for ongoing evaluation of Olyset Duo and PPF as a tool for controlling malaria mosquitoes and as a potential alternative for insecticide resistance management.

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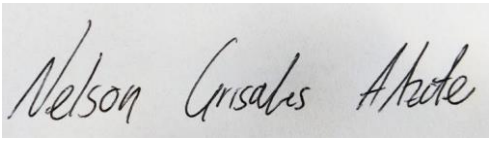
Declaration

I certify that this work has not been accepted in substance for any degree, and it is not currently being submitted other than that of Doctor in Philosophy being studied at the University of Liverpool. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised the work of others.

Signed: Date



8th September 2016

Nelson Grisales Alzate (Candidate)

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List of abbreviations

WHO	World Health Organization
DDT	Dichlorodiphenyltrichloroethane
ITN	Insecticide Treated Net
IRS	Indoor Residual Spraying
LLNI	Long Lasting Insecticidal Nets
PBO	Piperonyl butoxide
Kdr	knock-down resistance
CYP450	Cytochrome P450
COE	Carboxyl-Esterases
GST	Glutathione S-Transferases
UDP GT	UDP glucuronosyltransferase
ABC	ABC transporters
IGR	Insecticide Growth Regulator
PPF	Pyriproxyfen
JH	Juvenile Hormone
Duo	Olyset Duo
PPF net	1% pyriproxyfen net
BM	Bloodmeal
IB	Mosquitoes bloodfed immediately before exposure to a net
TTN	Through The Net (Mosquitoes bloodfed through a net)
CNFRP	Centre National de Recherche et de Formation sur le Paludisme
CI	Confidence Interval
EI	Emergence Inhibition
LSTM	Liverpool School of Tropical Medicine
LT50	Lethal Time 50
LC50	Lethal Concentration 50
BLR	Binary Logistic Regression
CDC	Centers for Disease Control and Prevention
OR	Odd Ratio
FC	Fold Change

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Chapter 1 Introduction and Literature Review

1.1 *Malaria history, distribution and incidence*

Malaria, a parasitic disease caused by the protozoan *Plasmodium*, is a burden humanity has endured for millennia. The employment of paleoparasitological tools determined that this disease was affecting humans as early as in the times of Neolithic dwellers, and as widespread as in Europe, Asia and Africa (Arrow et al., 2004).

Plasmodium falciparum, the etiological agent of severe malaria, is probably a descendant of *Plasmodium* species that infected early hominids in sub-Saharan Africa. It is believed that humans became hosts after a single cross-transmission event from gorillas (Liu et al., 2010, Prugnolle et al., 2011). It was the end of the nineteenth century that Charles Louis Alphonse Laveran, a French surgeon deployed in Algeria with the French Army, described the four different parasite forms in the blood of malaria infected patients. A couple of decades later Sir Ronald Ross, a Scottish physician in the Indian Medical Service, characterised the complete malaria life cycle in canaries demonstrating the mosquito involvement in the malaria parasitic cycle (Farrar et al., 2014).

In the first half of the twentieth century the greatest burden of malaria was in Asia (Carter and Mendis, 2002). However, a combination of political stability, the guidance of the newly founded World Health Organization (WHO) and the mass application of the organochlorine insecticide dichlorodiphenyltrichloroethane (DDT) contributed to a dramatic decline in malaria incidence from the 1950s (Carter and Mendis, 2002). However, the opposite was true in Africa where malaria cases rose in the latter half of the 20th century. In 2000, the United Nations (UN) included the fight against malaria as one of the Millennium development Goals (United Nations General Assembly, 2000). Partly as a result of this and other partnerships such as Roll Back Malaria, and the redesign and mass distribution of Insecticide Treated Nets (ITNs), there has been a global reduction on malaria cases and deaths (World Health Organization, 2015) (Figure 1.1).

In Africa it is estimated that the number of malaria cases declined from 321 (CI 253 - 427) per 1000 persons per annum

in 2000 to 192 (CI 135 - 265) per 1000 persons per annum in 2015 for a total reduction of 40% (Bhatt et al., 2015a); likewise the number of deaths declined from 764000 in 2000 to 395000 in 2015 (reduction of 48%) (World Health Organization, 2015).

P. falciparum and *P. vivax* are still globally distributed and co-occurring in most tropical and subtropical countries (World Health Organization, 2015). However some countries in the Americas, the Euro-Asian region, central Asia and the Koreas report only *P. vivax* malaria (Figure 1.2). The only country that currently report only *P. falciparum* malaria is the Central African Republic.

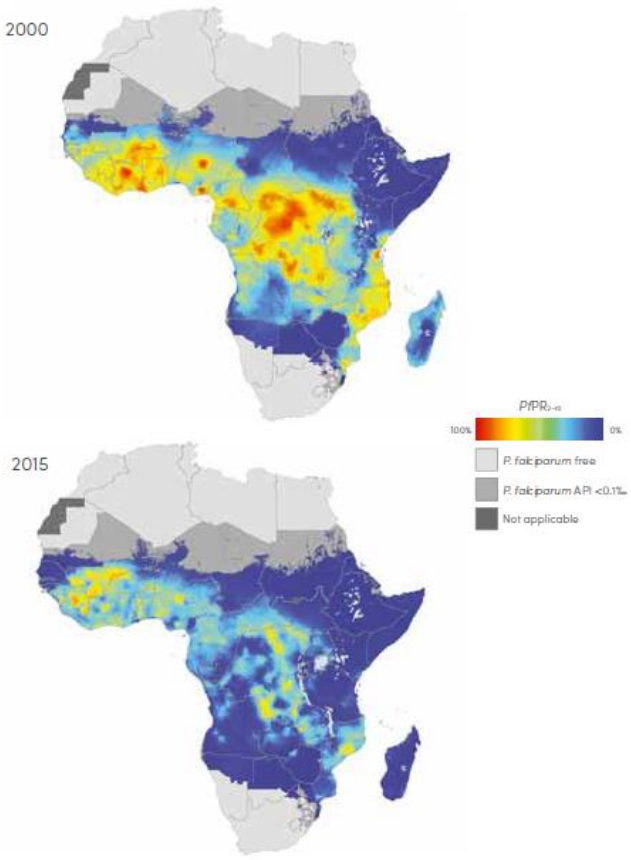


Figure 1.1 Plasmodium falciparum incidence in African children from 2000 to 2015 Estimated prevalence of *P. falciparum* on 2-10 years old children (PfPR$_{2-10}$) in Africa, between 2000 and 2015. API: Annual Parasite Index. Source: World Malaria Report 2015 (World Health Organization, 2015), from *Malaria Atlas Project* (Bhatt et al., 2015a).

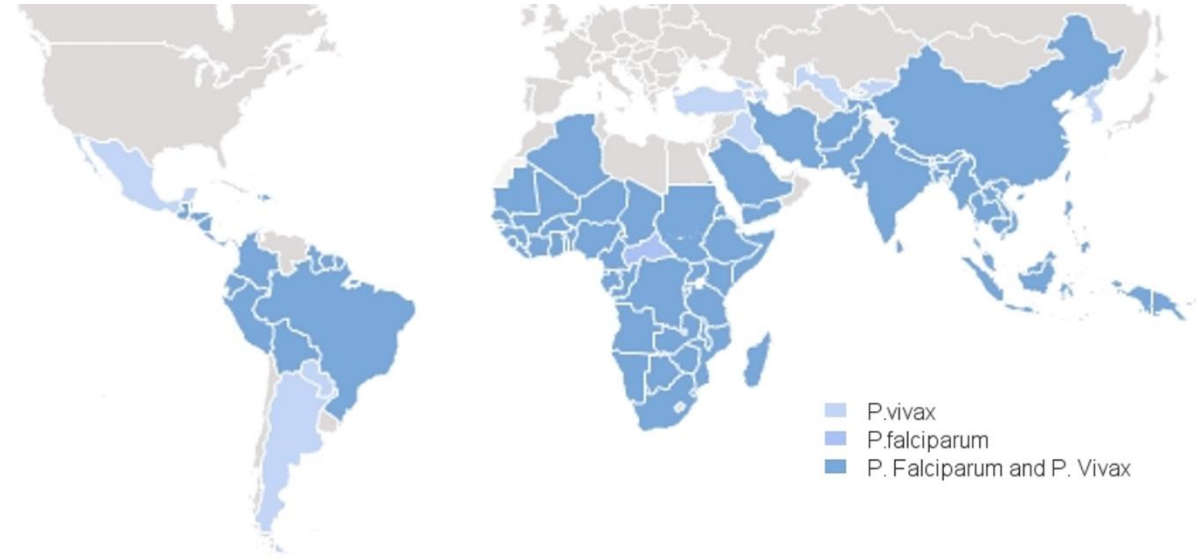


Figure 1.2 Distribution of *Plasmodium falciparum* and *Plasmodium vivax* Global distribution of *P. falciparum* and *P. vivax* according to the 2015 World Malaria Report. Map source: Global Malaria Mapper (http://www.worldmalariareport.org/).

1.2 *Malaria vectors*

The mosquitoes responsible for malaria transmission belong to the subfamily Anophelinae within the Culicidae dipteran family. From the three genera within this subfamily, only *Anopheles* and its subgenera *Kerteszia, Anopheles* and *Nyssorhynchus* are of medical importance (Manguin, 2013). In sub-Saharan Africa, *An. funestus* and three species belonging to the *An. gambiae* complex (*An. arabiensis, An. gambiae s.s.* and *An. coluzzii*) are widely distributed and are responsible for most of the malaria transmission (Figure 1.3). These species are frequently found sympatrically but have different behaviour and ecology, which influence the malaria transmission patterns.

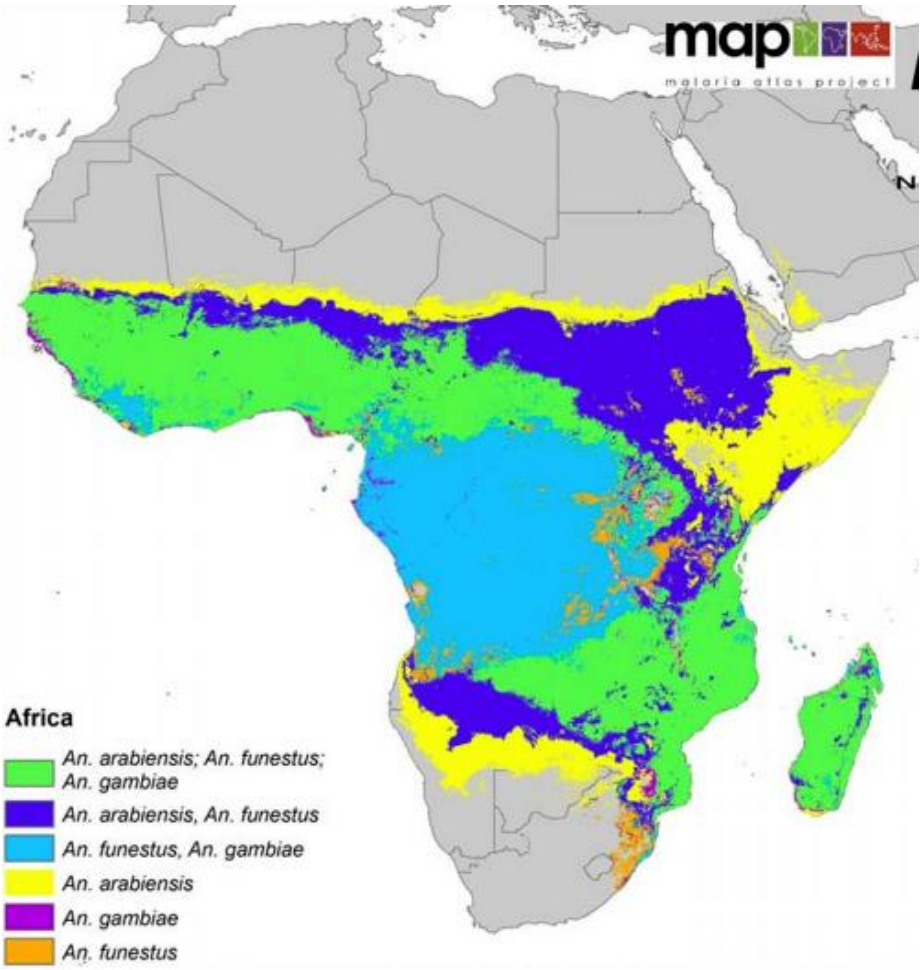


Figure 1.3 Distribution of medically important *Anopheles spp*. in Africa. The map shows the distribution of the three main species of anophelines responsible for malaria transmission: *An. funestus, An. arabiensis* and *An. gambiae* (now considered two separate species: *An. gambiae s.s.* and *An. coluzzii*). Map source: Sinka *et al.*, 2012 (Sinka et al., 2012).

1.3 *Anopheles gambiae sensu lato*

An. gambiae s.l. is a complex of morphologically indistinguishable species. *An. melas, An. merus* and *An. bwambae* breeds in salty water, therefore their distribution is limited to coastal regions, with *An. bwambae* highly endemic to Ugandan thermal springs. The freshwater species are *An. quadriannulatus*, which is considered a non-vector, and *An. arabiensis* and *An. gambiae s.s.,* which are widely distributed throughout Africa (White et al., 2011). *An. gambiae s.s.* was previously divided in two molecular forms, M and S characterised by low degrees of genetic differentiation (Favia et

al., 1997, della Torre et al., 2001, Gentile et al., 2001). These have recently been assigned species status with the former M molecular form now known as *An. coluzzii* and the former S form as *An. gambiae s.s.* (Coetzee et al., 2013). Additionally *An. amharicus*, described in Ethiopia, was also named as a separate species from *An. quadriannulatus* (Coetzee et al., 2013), bringing the total number of members of the species complex to eight. Three of these, *An. arabiensis*, *An. gambiae s.s.* and *An. coluzzii* are malaria vectors in Burkina Faso.

An. gambiae s.s. and *An. coluzzii* are highly anthropophilic, and have developed the ability to exploit a wide range of environments: for instance, mosquitoes can breed in human-made water reservoirs, dams, wells and rice paddies, and they also can rest inside houses or human constructions, obtaining more security against predators. They are also highly endophagic and endophilic (White, 1974).

Both species are sympatric in many regions of sub-Saharan Africa, but they also have different geographical and micro-geographical distributions (della Torre et al., 2005). *An. coluzzii* prefer stable and large breeding sites such as dams and rice paddies, while *An. gambiae s.s.* prefers temporary water bodies such as cattle tracks or holes alongside roads. It is likely that predation and the length of the rainy periods in different settings acted as an accelerator of divergence between these two species, contributing to the phenotypic differences of immature stages (Diabate et al., 2008).

An. arabiensis has greater phenotypic plasticity than *An. gambiae s.s.* or *An. coluzzii* and this is thought to be due to its abundance of chromosomal inversions (White, 1974). These mosquitoes have mixed preferences for biting animals and humans and they are frequently exophagic and exophilic, preferring biting and resting outdoors. Also, they can be more successful in arid, dry climates than the other species (White, 1974).

1.4 *Vector control*

Vector control is the primary method for prevention and control of malaria (Bhatt et al., 2015a). For several decades the main strategy of mosquito

control was Indoor Residual Spraying (IRS), until use of ITNs was scaled up in the 21st century.

IRS involves the spraying of insecticides on the walls of houses to reduce the lifespan and/or repel endophilic and/or endophagic mosquitoes. There are currently only four type of insecticides available for IRS: DDT, pyrethroids, organophosphates and carbamates. The first insecticide widely used in IRS was DDT which was deployed following the Second World War and its success contributed to the launch of the a campaign to eradicate malaria (Gahan et al., 1945). This insecticide was described not only as an effective insecticide, but also had excito-repellency properties that decreased human bite rates and shortened resting time indoors (Roberts and Andre, 1994). The emergence and global spread of DDT resistance coupled with serious ecological concerns contributed to a reduction in its use. Currently, this insecticide is still approved by WHO for use in IRS and is still used in some countries, although the majority have, or are in the process of, phasing out use of organochlorines (Sadasivaiah et al., 2007).

IRS programmes in Africa now more commonly use pyrethroids, the carbamate bendiocarb or the organophosphate pirimiphos methyl. Different parameters such as wall surface type, ph of the surface, temperature and humidity, as well as the formulation of the insecticide spray, affects the effectiveness of IRS programmes (Yeebiyo et al., 2016). Another issue is that in order to maintain effectiveness in some cases surfaces need to be sprayed twice/year. The biology and behaviour of the mosquitos targeted is also very important; IRS is effective against mosquitoes that bite and rest indoors, such as *An. gambiae s.s.* and *An. coluzzii*, but would be of little use for exophagic/exophilic species such as *An. arabiensis.*

IRS is still an important component of malaria control in some African countries (Sharp et al., 2007, World Health Organization, 2015) either being implemented by national programmes, the USA funded President's Malaria Initiative or, on a smaller scale, by private industry. However, in some countries resistance or suspicion of resistance to each of the four available insecticide families for IRS has been reported (Edi et al., 2012, Cisse et al., 2015, Keita et al., 2016). Furthermore the economic and operational

demands of maintaining IRS programmes are challenging for many African countries, and alternative malaria control tools are preferred. For example in Burkina Faso, a single pilot of IRS with bendiocarb was trialed in one district in 2013 but this was later abandoned due to high cost and limited efficacy. Today, only approximately 6% of those at risk of malaria in sub-Saharan Africa are protected by IRS (World Health Organization, 2015).

Bednets have been used to prevent mosquito biting for centuries, but it was only in the last century that these were treated with insecticides. By the end of the 1980s bednets impregnated with permethrin were tested in different locations and the results consistently showed them to be effective in preventing malaria (Graves et al., 1987, Lines et al., 1987, Lindsay et al., 1989). Initially the insecticide on an ITN was applied by dipping but it was relatively unstable requiring re-treatment at least every six months. This problem was solved with the development of Long-lasting Insecticidal Nets (LLINs) that maintained high and stable insecticide concentrations without any need of re-treatment (Jamison et al., 2006). It was the development and mass distribution of these LLINs that marked the beginning of a period of malaria decline in Africa. The proportion of people sleeping under the protection of bednets in sub-Saharan Africa was less than 2% in 2000 and increased to 55% by 2015, contributing to a reduction of 68% of *P. falciparum* prevalence (Bhatt et al., 2015a). ITNs have also been attributed with a reduction in clinical episodes of uncomplicated malaria caused by *P. falciparum* and *P. vivax* by 50% (range 39–62%), as well as reducing the prevalence of high-density parasitaemia (Bhatt et al., 2015a).

All LLINs currently in use are either coated or impregnated with pyrethroids (permethrin, deltamethrin or alpha cypermethrin). More recently the pyrethroid synergist piperonyl butoxide (PBO) has been added to some nets in an attempt to increase their efficacy against pyrethroid resistant populations (Tungu et al., 2010, Pennetier et al., 2013). New combination nets containing either pyrethroid plus chorfenapyr or pyrethroid plus pyriproxyfen are currently under WHO evaluation (N'Guessan et al., 2014, Tiono et al., 2015).

1.5 *Insecticide resistance*

The Insecticide Resistance Action Committee (IRAC) defines insecticide resistance as 'the selection of a heritable characteristic in an insect population that results in the repeated failure of an insecticide product to provide the intended level of control when used as recommended' (IRAC, 2011). The first documented case of insecticide resistance was reported in 1947, when mosquitoes from the genus *Aedes spp.* showed tolerance to DDT in the United States (Brown, 1986). Since then, resistance to all approved chemical insecticides has been reported in the main mosquito species of public health importance. In malaria mosquitoes, insecticide resistance to all four types of insecticides used to target adults has emerged as the consequence of the strong selective pressure caused by the escalation of malaria control programmes (World Health Organization, 2012).

Resistance to pyrethroids in malaria vectors in Africa was first reported by Davidson and Curtis in 1978 (Davidson and Curtis, 1978) and is now very widespread across Africa (Ranson and Lissenden, 2016). In West Africa, where the current study was conducted, resistance to pyrethroids has been described in Mali (Cisse et al., 2015), Nigeria (Awolola et al., 2002, Okorie et al., 2015), Benin (Djegbe et al., 2011, Gnanguenon et al., 2015), Ivory Coast (Chandre et al., 1999, Koffi et al., 2013), The Gambia (Tangena et al., 2013), Liberia (Temu et al., 2012), Burkina Faso (Toe et al., 2014) and Ghana (Adasi and Hemingway, 2008). Although susceptible populations can still be found (Opondo et al., 2016), as resistance sweeps across the region these may soon disappear.

The diagnostic or discriminating concentration of an insecticide is defined as the concentration that kills twice 99.9% of a susceptible mosquito population ($LC_{99.9}$) (World Health Organization, 2013). If the mortality of a mosquito population is lower than 90%, insecticide resistance is diagnosed. However, this strategy fails to provide information of the strength of the resistance (Bagi et al., 2015). Bioassays measuring mortality resulting from exposure to different ranges of times and concentrations have proved more informative in characterising the strength of insecticide resistance in highly

resistant mosquito populations (Toe et al., 2014, Bagi et al., 2015, Etang et al., 2016).

1.6 *Insecticide resistance mechanisms*

Insecticide resistance mechanisms have been classically divided into four types: target site mutations, metabolic resistance, reduced penetration and behavioural resistance. Target site mutations comprise any alteration in the site where the insecticides bind, reducing or preventing the interaction. Mutations in the domain II region of the *para*-type Voltage-gated Sodium Channel (VGSC), the site of action of DDT and pyrethroids are termed kdr mutations (from knock-down resistance). In *An. gambiae s.l.* the most common kdr mutations are L1014F (referred to in the earlier literature as the West African kdr) and L1014S (previously known as the East African kdr) (Martinez-Torres et al., 1998, Ranson et al., 2000). An additional mutation (N1575Y) within the linker between domains III-IV, which is only found on a 1014F haplotype, confers higher resistance levels (Jones et al., 2012). The distribution and variations of kdr mutations in *Anopheles spp.* were recently reviewed by Silva *et al*. (Silva et al., 2014).

Other target site mutations responsible for insecticide resistance include modifications in the *ace-1* and the *Rdl* (Resistance to dieldrin) genes. In *An. gambiae,* the insensitive acetylcholinesterase (iAChE) phenotype that confers resistance to OPs and carbamates results from the G119S mutation in that gene (Weill et al., 2002, Weill et al., 2004). Alternative mutations in *ace-1* have been reported for *An. albimanus*, a primary malaria vector in the New World (Liebman et al., 2015b). Mutations in the Rdl gene which encodes the ɣ-aminobutyric acid (GABA), particularly A296G in *An. gambiae* and A296S in *An. arabiensis*, are responsible for resistance to dieldrin (Du et al., 2005).

Metabolic resistance is related to the overexpression or enhanced performance of detoxification enzymes in sequestering or metabolising the insecticide molecules before they reach their target. The overexpression can occur as a result of enhanced upregulation or the multiplication of the physical copies of the detoxification gene. The enhanced performance can

occur by mutations that modify the structure of the enzymes making them more efficient in binding and processing the insecticide substrate. There are three detoxification families encompassing most of the metabolic mechanisms of insecticide detoxification: Cytochrome P450s (CYP450s, also known as Mixed Function Oxidases), Carboxyl-Esterases (COEs) and Glutathione S-Transferases (GSTs). Growing evidence also indicates that two additional gene families, UDP glucuronosyltransferases (UDP GTs) and ABC transporters (ABCs), are also involved in insecticide resistance (Fossog Tene et al., 2013, Epis et al., 2014, Ishak et al., 2016).

Target site resistance mutations are highly conserved across the insect species because the insecticide target sites are structural part of the nervous system, which are highly sensitive to non-synonymous modifications altering functions (Silva et al., 2014). This has facilitated the design of diagnostic markers useful to track these resistance mechanisms in the mosquito populations. However, due to the high diversity and independent evolution of detoxification genes, it has been difficult to design these kind of markers for metabolic resistance. Several candidate genes for insecticide resistance and cross-resistance to multiple insecticide classes, including CYP6P3, CYP6P9, CYP6M2, CYP6Z2 and GSTE2, have been characterised (Muller et al., 2007, Djouaka et al., 2008, Muller et al., 2008, Mitchell et al., 2012, Fossog Tene et al., 2013, Ingham et al., 2014, Matowo et al., 2014, Riveron et al., 2014, Bonizzoni et al., 2015, Ibrahim et al., 2015). Furthermore, *in vitro* and *in vivo* experiments have confirmed that some of those genes are efficient at metabolising insecticides (Daborn et al., 2012, Mitchell et al., 2012, Edi et al., 2014, Mitchell et al., 2014). However, as yet, very few molecular markers for metabolic resistance exist (Donnelly et al., 2016).

Reduced insecticide penetration can occur as a result of a thickened cuticle or a cuticle with a modified composition. Although differential expression of different cuticular proteins has been observed in resistant mosquito populations (Balabanidou et al., 2016), their direct involvement on insecticide resistance is still being researched. Recently some of the cuticular proteins over transcribed in resistant populations were localised to

the mosquito appendages; this is supportive of a role in insecticide resistance as tarsal uptake is the main vehicle of insecticide entering the mosquito (Vannini et al., 2014).

Behavioural resistance is described as any change in mosquito behaviour that reduces the chances of contact with the insecticide in comparison with the behaviour normally observed in the species. For example the use of IRS with DDT has been linked to a shift from biting indoors to outdoors or to earlier biting times (Charlwood and Graves, 1987, Lindsay et al., 1993, Takken, 2002, Gatton et al., 2013). Another possible behavioural change is the preference for more easily accessible hosts (such as the ones found outdoors). Understanding these potential behavioural shifts is key for the success of malaria eradication goals. A good example is that is the case of vector control in South West Asia where DDT-IRS was able to practically eliminate two important malaria vector mosquito populations, but a remaining third species (*An. farauti*) adapted its host-seeking behaviour to bite early in the evenings and outdoors, maintaining the malaria levels in the region (Russell et al., 2013).

1.7 *Insecticide resistance impact on malaria control*

It is not clear if insecticide resistance is reducing the protection conferred by LLINs against malaria infection. Although insecticide resistance has been linked with a reduction in the effectiveness of LLINs (Toe et al., 2014, Bagi et al., 2015), studies have shown that the distribution of ITNs on pyrethroid-resistant areas is still effective in protecting humans (Lengeler, 2004, Henry et al., 2005, Damien et al., 2010, Tokponnon et al., 2014, Lindblade et al., 2015). Recently, Viana *et al*. reported that cumulative exposure to insecticides leads to a shorter lifespan in multi-resistant mosquitoes, hypothesising that this may explain the success of LLINs observed in the field in diminishing malaria transmission rates (Viana et al., 2016). Another possible factor is that although resistant mosquitoes may not be killed by the insecticide in the LLINs, they would be prevented to bite by the pyrethroids excito-repellent properties; however in this case the community effect, which is the indirect protection granted by LLINs to non-covered

people via mosquito mortality, would be seriously compromised (Lindblade et al., 2015).

Longitudinal interventions have shown the impact of insecticide resistance on malaria. In Uganda, the shift from DDT to bendiocarb in IRS interventions against DDT-resistant carbamate-susceptible mosquitoes caused a reduction in malaria morbidity in patients <5 years old (Kigozi et al., 2012). Malaria incidence in South Africa was associated with the development of deltamethrin resistance in *An. funestus* (Hargreaves et al., 2000). In the case of LLIN, some studies have failed to demonstrate the link between LLNI usage and reductions in malaria. In Tororo, Uganda, where *Anopheles gambiae s.l.* shows pyrethroid resistance, LLINs coverage of up to 62% for children < 5 years old in combination with Artemisin-based combination therapy (ACT) failed to impact malaria morbidity (Jagannathan et al., 2012). Similar results were reported in Burkina Faso (Louis et al., 2015), Mali (Coulibaly et al., 2014) and Malawi (Roca-Feltrer et al., 2012). Although the current impact of insecticide resistance on malaria control interventions remains controversial, insecticide resistance is clearly a future threat that highlights the need for new control tools.

1.8 *Insecticide resistance management*

Malaria control in Africa is heavily dependent on the effectiveness of pyrethroids and therefore resistance to this insecticide class is a risk factor for sustained control. In 2012 the WHO developed the Global Plan for Insecticide Resistance Management (GPIRM) (World Health Organization, 2012), identifying a set of steps to achieve a sustainable global strategy to reverse or halt the insecticide resistance intensity in the short, medium and long term. The basis of the plan resides in planning and implementing insecticide resistance management strategies, which requires robust entomological surveys of insecticide resistance, development of new vector control tools, the completion of knowledge gaps about insecticide resistance mechanisms and advocacy to ensure that human and financial resources are available (World Health Organization, 2012). Strategies to manage insecticide resistance are context specific, and must consider factors such

as the behaviour of the target mosquitoes, the mechanisms of insecticide resistance, the selective pressure not only by public health insecticides but also by agricultural pesticides and other pollutants, the budget, infrastructure and other logistical limitation of the local authorities and human acceptance of the control methods. For IRS insecticide resistance management can involve the rotation of different classes of insecticides or as mosaic applications or the deployment of interventions in combination (*i.e.* IRS and LLINs) (World Health Organization, 2012). These strategies aim to combine insecticides with different modes of action to avoid cross-resistance; the weakness is that currently several mosquito populations already possess different mechanisms of resistance (Dabire et al., 2008, Edi et al., 2014, Ibrahim et al., 2016). Furthermore implementing these strategies is often challenging in African settings. Insecticide resistance management in the context of LLIN programs offers limited choices, given the current reliance on a single insecticide class. The development of LLINs that can incorporate insecticides with new modes of action are essential for the sustainability of those vector control programs. Without a commercial alternative to pyrethroids on LLINs, it can only be expected that the mosquito populations will develop increasing levels of resistance.

New chemistries for malaria vector control have become available over the past decade. These include new formulations of organophosphate insecticides for long lasting IRS (N'Guessan et al., 2010, Rowland et al., 2013), plastic sheets treated with insecticides (Graham et al., 2002, Mittal et al., 2011, Burns et al., 2012) and paints with a combination of organophosphates and an Insecticide Growth Regulator (IGR) (Mosqueira et al., 2010a, Mosqueira et al., 2010b); molecules re-purposed from agriculture such as neonicotinoids (Corbel et al., 2004, Allan, 2011, Uragayala et al., 2015), the pyrrole insecticide chlorfenapyr (Raghavendra et al., 2011, Verma et al., 2015) and the entomopathogenic fungi *Beauveria bassiana* (Farenhorst et al., 2009, Howard et al., 2010). Two types of combination nets, both containing pyrethroids are now available or expected to be shortly on the market: synergist nets such as Permanet 3 (deltamethrin and the synergist PBO in the roof panel) (Koudou et al., 2011,

Awolola et al., 2014, Abilio et al., 2015), Olyset Plus (permethrin and PBO) (Pennetier et al., 2013) and the Interceptor G2 nets treated with chlorfenapyr and a pyrethroid (N'Guessan et al., 2014).

1.9 *Juvenile Hormone and Pyriproxyfen*

The Juvenile Hormone (JH) (Figure 1.4) is an essential molecule expressed differentially during different phases of the mosquito life cycle, and is involved in development, behaviour, reproduction, diapause and metamorphosis (Wilson, 2004). Its presence-absence at different levels trigger different processes; for example, JH stimulates the larval develop process across the different stages, but its titres must be low to null to allow metamorphosis to pupae (Wilson, 2004).

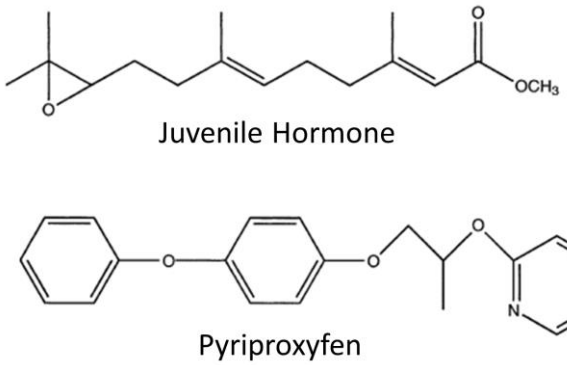


Figure 1.4 Chemical structure of Juvenile Hormone and Pyriproxyfen. Figure taken from Wilson, 2004 (Wilson, 2004).

The JH plays a crucial role in insect reproduction too. The endocrine pathways of insect reproduction are governed by three kind of molecules: neuropeptides, ecdysteroids (particularly the 20 hydroxyecdysone – 20E) and juvenile hormones. In mosquitoes and other Diptera the 20E hormone has a prominent role in the hormonal regulation of reproduction, while the JH function is more specialised in preparing 20E-mediated events such as vitellogenesis (the synthesis of yolk) and reproductive tissues (Gilbert et al., 2005). Bloodmeal signals the stop of JH synthesis and trigger the secretion of JH-esterases and ecdysone, which is transformed in 20E; this hormone then stimulates the vitellogenin production by the fat body. Vitellogenesis has four phases: previtellogenic preparation, arrest, yolk protein synthesis

(*i.e.* vitellogenesis) and termination (Tobe et al., 1994). The first stage, regulated by JH, starts immediately after the female has emerged and come to a halt (arrest phase) approximately 3 days after, until a bloodmeal is taken. When this happens the 20E stimulates the production of yolk that is accumulated by the developing oocytes, peaking 24 h after the bloodmeal; eventually the chorion formation is completed and the eggs can be oviposited (Gilbert et al., 2005). Because of the JH role in preparation and the 20E role in vitellogenesis, the hormones show contrasting activity peaks before and after bloodmeal (Figure 1.5).

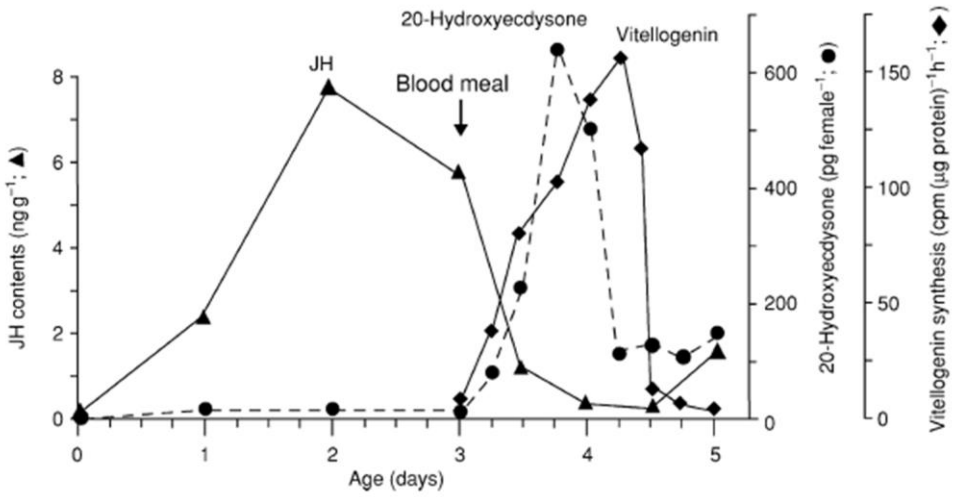


Figure 1.5 Levels of Juvenile Hormone (JH), 20 hydroxyecdysone and Vitellogenin in Aedes aegypti before and after a bloodmeal. Image taken from Gilbert *et al.,* 2005 (Gilbert et al., 2005).

Pyriproxyfen (4-phenoxyphenyl (RS)-2-(2-pyridyloxy)propylether) (PPF) is a JH analogue. Although the chemical structures differ (Figure 1.4), PPF is a powerful JH agonist (Hatakoshi et al., 1986, Hatakoshi et al., 1988, Riddiford and Ashburner, 1991). PPF affects metamorphosis and oogenesis in insects, but a unique mode of action have not been characterised yet. The reason for this is that the studies done in different insect species does not always agree about the specific process that is disturbed by PPF. For example, PPF caused lack of yolk deposition in eggs laid by the cat flea *Ctenocephalides felis* (Palma et al., 1993), but arrested embryo development in *Aedes spp.* mosquitoes (Xu et al., 2015). In *An. gambiae*

PPF impairs the development of the ovarian follicles at some point between the previtellogenic preparation stage and the termination stage, in a process that probably inhibits the production of ecdysone (Koama et al., 2015).

PPF is already used as a larvicide to control mosquitoes in the form of SumiLarv (Sumitomo Chemicals Ltd) (World Health Organization, 2001, Mbare et al., 2013). It is very effective at low concentrations, but its effectiveness decreases inversely to the amount of organic material in the water. Pilot studies on malaria vectors in Kenya have found that wild mosquitoes are fully susceptible to the operational dose under standardized field conditions (Mbare et al., 2013). Currently, SumiLarv is not used operationally for malaria control. No cross-resistance with other insecticides was reported in mosquitoes prior to this study (Kawada et al., 1993); however, new insights on this topic were addressed in this thesis.

Mosquitoes can transport PPF crystals adhered to their appendages between breeding sites. This autodissemination has been proven effective in field trials of *Aedes aegypti* (Itoh et al., 1994, Devine et al., 2009, Suarez et al., 2011) because this mosquito species prefer artificial, relatively small breeding sites that makes the PPF concentration higher when the crystals are transferred. Small scale trials in Sri Lanka have also shown a negative impact on mosquito density and malaria prevalence after an intervention with PPF (Yapabandara et al., 2001).

JH analogues also impair the reproductive success of mosquitoes by affecting the oogenesis (Patterson, 1974, Loh and Yap, 1989). Although the information about the specific effect of PPF on adult malaria vectors is not consistent across scientific literature, studies agree that PPF reduces reproductive output (Aiku et al., 2006, Ohashi et al., 2012, Harris et al., 2013) and reduces adult longevity (Ohashi et al., 2012, Kawada et al., 2014).

Sumitomo Chemicals Ltd have developed a LLIN (Olyset Duo) combining permethrin and PPF. The rationale behind this innovative LLIN is this: in a scenario where a susceptible mosquito come in contact with the net, the effect of the permethrin would be lethal; but if the mosquito is resistant to

permethrin, the PPF acquired should sterilise it therefore preventing the perpetuation of the insecticide resistant genes, and hypothetically reducing the mosquito density in the area. Recently, semi-field evaluations of Olyset Duo (Duo) have been carried out across Africa with mixed results. Release-recapture hut trials that showed high sterilisation rates of susceptible and resistant laboratory mosquitoes in Benin (Djenontin et al., 2015). Also, experimental hut trials in the same country showed either reductions in fecundity or complete sterilisation of mosquitoes due to Duo and PPF LLINs (Kawada et al., 2014, Ngufor et al., 2014). However hut trials in an areas of Côte D'Ivoire with multi-resistant *An. gambiae* found a significant effect on mosquito fertility but no impact on fecundity (Koffi et al., 2015). Some logistical challenges such as the low survival of mosquitoes up to the oviposition stage and low oviposition rates in wild *Anopheles spp.* mosquitoes were evident in these studies, resulting in low sample sizes. A small scale field trial of Duo reported a reduction in the number of fertile females and in the reproductive output of those mosquitoes not fully sterilised but again the sample size was very small (Kawada et al., 2014). The implementation of a full field trial on Duo was strongly recommended to obtain better quality data on entomological and clinical aspects.

1.10 *Study site and Olyset Duo clinical trial*

Burkina Faso is a landlocked West African country. It has borders with Mali in the north and west, Niger in the east and Cote d'Ivoire, Ghana, Togo and Benin in the south (Figure 1.6). It has a bimodal climate, with a dry season roughly between October and May and a rainy season between June and September, and annual rainfall of more than 500 mm (U.S. Geological Survey, 2012). Around 80% of the population depends on agricultural activities (U.S. Geological Survey, 2012), which are mostly developed during the rainy season and focus on cotton, maize, sorghum and millet (Food and Agriculture Organization of the United Nations et al., 2014). The Gross Domestic Product (GDP) per capita is one of the lowest in the world and the population growth average is one of the highest (Food and Agriculture Organization of the United Nations et al., 2014); this, added to

poor infrastructure, recent political instability and climatic factors, make Burkina Faso a specially susceptible country to tropical diseases.

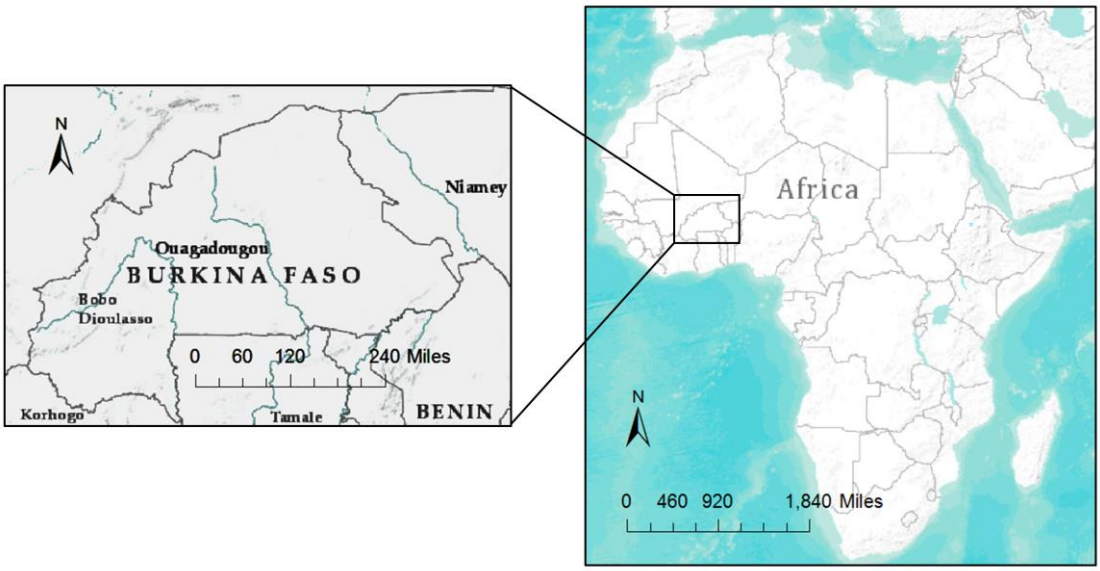


Figure 1.6 Burkina Faso location. Map showing the location of Burkina Faso in West Africa.

Burkina Faso is a high malaria transmission country (World Health Organization, 2015), and this disease is the main cause of severe illness and death among children (Tiono et al., 2014). Universal LLIN coverage campaigns took place in 2010 and 2013 aiming to distribute one LLIN per two people in households (Zollner et al., 2015). However, it was reported that the campaign did not achieve the goal of conferring better protection to children younger than 5 years old (Louis et al., 2015).

In recent years an abrupt increase in the prevalence of pyrethroid resistance across the country have been described (Namountougou et al., 2012). In the south west pyrethroid resistance levels >1000 fold higher than susceptible populations have been reported (Toe et al., 2014). The cause of the resistance in these mosquitoes may not only be because of the massive selective pressure from LLINs, but also because of insecticides used in agriculture practices (Diabate et al., 2002). Studies on the mechanisms of insecticide resistance in mosquitoes from Vallee du Kou (Bobo Dioulasso) and Tengrela (Banfora) (Kwiatkowska et al., 2013, Toe et al., 2015) identified a range of candidate genes whose expression was associated

with insecticide resistance. Target site resistance (kdr 1014F) is also very prevalent in the southwest of the country (Toe et al., 2015). The level of resistance in this region of the country has been shown to render the insecticide on LLINs ineffective in laboratory assays (Toe et al., 2014).

There is therefore an urgent need for new tools that can control pyrethroid resistant populations in this study site. In 2014 and 2015 a step-wedge design, randomised controlled trial was implemented in Banfora district to determine whether Duo provides additional protection against clinical malaria over standard Olyset nets (Tiono et al., 2015). The results in this thesis describe a complementary series of experiments to assess the impact of Duo LLINs on the local vector population. The specific objectives were:

1. To investigate the effect of pyriproxyfen on the longevity, reproductive output and metamorphosis of insecticide resistant and susceptible laboratory mosquitoes.
2. To describe and monitor the permethrin resistance intensity and mechanisms in the local Anopheles populations for the duration of the Olyset Duo Randomised Controlled Trial in Burkina Faso.
3. To assess the effect of Olyset Duo on the reproductive output of wild malaria mosquitoes during the Randomised Controlled Trial in Burkina Faso.

Chapter 2 Effect of exposure to pyriproxyfen-treated nets on *Anopheles gambiae* fitness traits

2.1 *Introduction*

The Juvenile Hormone (JH) is involved in several physiological events during the mosquito's life cycle. Its absence or presence acts as a signal to stop or to trigger different processes including adult emergence and egg production and thus analogues of JH have multiple potential applications in mosquito control. The JH analogue pyriproxyfen (PPF) is approved by WHO as a larvicide (World Health Organization, 2001). A key advantage of JH analogues is that they are active at extremely low concentrations and studies on *Aedes aegypti* have shown that mosquitoes are able to transfer sufficient PPF between breeding sites to autodisseminate the insecticide (Caputo et al., 2012). Proof of principle trials have also shown the potential for malaria mosquitoes to transfer PPF between breeding sites in semi field systems although not, as yet, under natural settings (Itoh et al., 1994, Dell Chism and Apperson, 2003, Sihuincha et al., 2005, Devine et al., 2009, Lwetoijera et al., 2014b, Mbare et al., 2014).

In addition to disrupting metamorphosis, JH analogues also can permanently block follicular development in female mosquito ovaries (Judson and de Lumen, 1976) and reduce the adult lifespan. However, the potential of the effects of PPF on the adult population for mosquito control have received less attention. Recently, Sumitomo Chemical Ltd introduced a modified form of its LLIN Olyset, which, in addition to the permethrin contained in Olyset nets also contained PPF (Ngufor et al., 2014). Laboratory and experimental hut trials of these Olyset Duo Nets, or nets containing PPF alone, showed that *An. gambiae s.l.* mosquitoes exposed to PPF containing nets were sterilised and had reduced longevity although the magnitude of these effects varied between studies and, in some cases, was dependent on the stage of the gonotrophic cycle at which exposure occurred (Table 2.1).

In *Ae. aegypti* JH titres start decreasing immediately after a bloodmeal (BM) reaching the lowest point 48 hours after it; then JH increase steadily until, after around 69 hours, the levels are the same as before the BM (Shapiro et al.,

1986) (Table 2.1). This would suggest that the optimal time of exposure to PPF to maximize the impact on mosquito reproduction would be *after* a BM. Given that female mosquitoes are more likely to encounter PPF on a bednet when actively host seeking i.e. *before* taking a blood meal this is a potential limitation of the Olyset Duo paradigm. However, although greater impact of PPF encountered after a BM is supported by Patterson (1974) and more recently Harris et al (Patterson, 1974, Harris et al., 2013), other studies show that exposures before a BM can be also very effective (Table 2.1). One of the limitations of those studies that makes the determination of the most effective time to expose mosquitoes to PPF relative to a bloodmeal is the methodological differences and lack of a specific period of time between PPF exposure and bloodmeal. For example, Ohashi *et al.* left mosquitoes to bloodfeed overnight, therefore the contact with PPF could have been immediately before/after or around 6 hours before/after the bloodmeal (Ohashi et al., 2012) (Table 2.1).

The purpose of this chapter was to determine the impact of exposure of *An. gambiae* to the concentration of PPF used in Olyset Duo on adult longevity and egg production and viability, and to determine whether the magnitude of this effect was dependent on the stage of the gonotrophic cycle in which exposure occurred. These laboratory experiments were necessary to help interpret results from field evaluation of Olyset Duo nets described in subsequent chapters.

Cone bioassays are typically used to measure the response of mosquitoes to insecticide impregnated materials (World Health Organization. Dept. of Control of Neglected Tropical Diseases. and WHO Pesticide Evaluation Scheme., 2006). Our own personal observation, and those of others (Angela Hughes person. comm.) have found that the duration of mosquito contact with the insecticide-treated net in this assay are variable, leading in some cases to the underestimation of the tested molecule efficacy; this especially applies for molecules with excito-repellent properties. A modified version of a cone bioassay was therefore trialled in this chapter which utilized a deli-pot.

Table 2.1 Overview of previous studies assessing the impact of pyriproxyfen on the reproductive output of mosquitoes. All exposure times were for 3 minutes, except in Harris (2013) where the exposure was done for 30 minutes. $^{+}$'X' refers to an unknown measure of time. In these experiments, mosquitoes were left overnight with a host to feed freely.

Publication	Mosquito species	Method of exposure	JHA	Timing of PPF exposure relative to blood-meal	Effect
(Shapiro et al., 1986)	*Ae. aegypti*	Topical	100 pg Methoprene/µl	30 hours after	<Fertility
(Ohashi et al., 2012)	*An. gambiae*	Tarsal (net)	PPF 0.1%	X^{+} hours before	<Fecundity, < Egg hatchability
				X^{+} hours after	<Fecundity, < Egg hatchability
			PPF 0.01%	X^{+} hours before	<Fecundity, <Egg hatchability
				X^{+} hours after	<Fecundity, < Egg hatchability
			PPF 0.001%	X^{+} hours before	<Proportion egg-laying, =Fecundity, = Egg hatchability
				X^{+} hours after	=Proportion egg-laying, =Fecundity, < Egg hatchability
(Harris et al., 2013)	*An. arabiensis*	Tarsal (impregnated glass bottle)	PPF 3mg/m^2	3 days before	=Fecundity, = Egg hatchability
				1 day before	=Fecundity, = Egg hatchability
				1 day after	<Fecundity, No Egg hatchability
				3 days after	=Fecundity, = Egg hatchability
(Mbare et al., 2014)	*An. gambiae*	Tarsal (impregnated plastic); 30 minutes	PPF 2.6 mg/m^2 (in SumiLarv®)	2 days before	<Proportion egg-laying, <Fecundity, <Egg hatchability
				1 day before	<Proportion egg-laying, <Fecundity, <Egg hatchability
				30 minutes before	<Proportion egg-laying, <Fecundity, <Egg hatchability
				30 minutes after	<Proportion egg-laying, <Fecundity, <Egg hatchability
,				1 day after	<Proportion egg-laying, <Fecundity, <Egg hatchability
				2 days after	=Proportion egg-laying, =Fecundity, =Egg hatchability
				3 days after	=Proportion egg-laying, =Fecundity, =Egg hatchability

The objectives of this chapter were:

1. To measure the effect of PPF exposure on mosquito reproductive output across multiple gonotrophic cycles.
2. To determine the optimal timing of mosquito-PPF contact in relation to bloodmeals.
3. To assess the impact of PPF exposure on mosquito longevity.

All experiments were performed on an insecticide susceptible strain of *An. gambiae* and PPF exposure was via tarsal contact with a PPF impregnated net provided by Sumitomo Chemicals Ltd.

2.2 *Methods*

An. gambiae Kisumu strain were reared in the insectary of the Centre National de Recherche et de Formation sur le Paludisme, (CNFRP) in Ouagadougou, Burkina Faso, with an average temperature of 27°C – 30°C, relative humidity of 75% - 95% and a photoperiod of 12 hours light/12 hours dark. Larvae were fed with TetraMin Baby® fish food. For all the experiments, 3 days-old females which had been provided with sucrose ad libitum were used. A 1% pyriproxyfen treated net (PPF net) was provided by Sumitomo Chemical Co. LTD. (Tokyo, Japan). Experiments took place between May-September of 2013.

2.2.1 Timing of exposure to pyriproxyfen relative to blood-meals

To investigate the effect of timing of PPF exposures relative to the bloodmeal (BM), five alternative treatments were studied (Figure 2.1). The selection of these treatments was based on methodologies already published (see Table 2.1), but also based on potential real life mosquito – net contact scenarios. Mosquitoes were exposed to PPF 24 hours (-24h), 6 hours (-6h) and immediately before the BM (IB), during a BM (Through-the-net, TTN) and 24 hours after a BM (+24h) (Table 2.2). The -24h experiment reflects the possibility of mosquitoes getting exposed to the PPF while trying to bloodfeed, and being successful one day (24 hours) after that. The -6h experiment aimed to emulate

an initial contact with the PPF net that prevent biting but eventual success by the end of the night. IB reflect the possibility that mosquitoes succeed biting shortly after contacting the net, and TTN reflect the possibility of mosquitoes biting through it. Finally, +24h was considered as an additional treatment that could be informative as it has been effective in other studies (Harris et al., 2013, Mbare et al., 2013) (Table 2.1). Every treatment was repeated with an untreated net as a negative control. Mosquitoes in all treatments were 3 days old at the time of BM, except in the -24h cohort where PPF exposure occurred on day 3 and the BM was offered the following day, when mosquitoes were 4 days old. An additional treatment where the mosquitoes were exposed to PPF but not bloodfed (NBM) was also included.

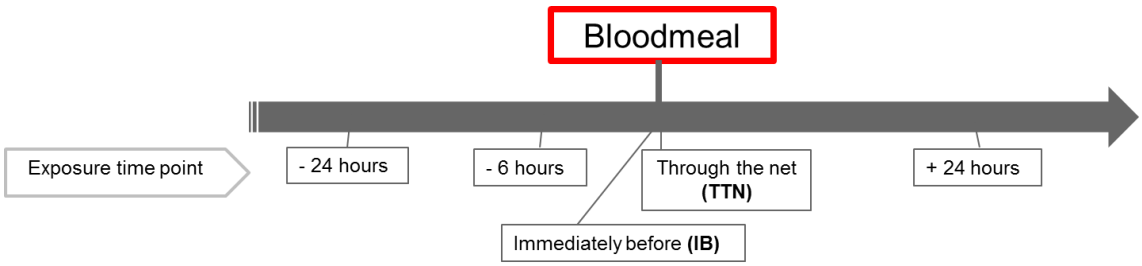


Figure 2.1 Schematic representation of the different treatments selected to evaluate the impact of a single pyriproxyfen exposure before, during and after bloodmeal. -24/-6 hours: mosquitoes exposed to PPF 24/6 hours before BM; Immediately before (**IB**): mosquitoes were exposed and immediately bloodfed; through the net (**TTN**): mosquitoes were engorged on a rabbit through a piece of PPF or untreated-net; and **+24 hours**: mosquitoes were exposed to PPF 24 hours after BM.

Table 2.2 Abbreviation of each pyriproxyfen exposure time relative to a bloodmeal.

Abbreviation	PPF exposure time relative to bloodmeal
-24h	24 hours before
-6h	6 hours before
IB	Immediately before
TTN	Simultaneous
+24h	24 hours after
NBM	Without bloodmeal

Two different experimental designs were applied to 1) determine the effect of PPF on adult survival and lifelong fecundity, and 2) to measure the impact of PPF on the fecundity and fertility of individual mosquitoes.

2.2.2 Survival and lifelong oviposition

2.2.2.1 Deli pot bioassay

Mosquitoes were exposed to a piece of the nets using the Deli pot bioassays. 25 ml pots (height 28 mm, top diameter 50 mm base diameter of 40 mm, supplied by Cater for you LTD (High Wycombe, UK)) were prepared by cutting a large whole in the lid of the pot and a smaller (approximately 1 cm diameter) hole in the bottom (Figure 2.2A). The lid and the pot were assembled with a piece of the untreated/PPF-net between them (Figure 2.2B). According to each treatment, groups of ten mosquitoes were introduced by manual aspirator to the assembled plastic pot exposing to the PPF/untreated net for three minutes and then transferred to a 30x30x30 cm mosquito cage with sucrose solution. Mosquitoes from up to ten replicate exposures were combined in single cages, according to availability (Table 2.3). In each cage 3-5 males were left in an attempt to increase copulation rates.

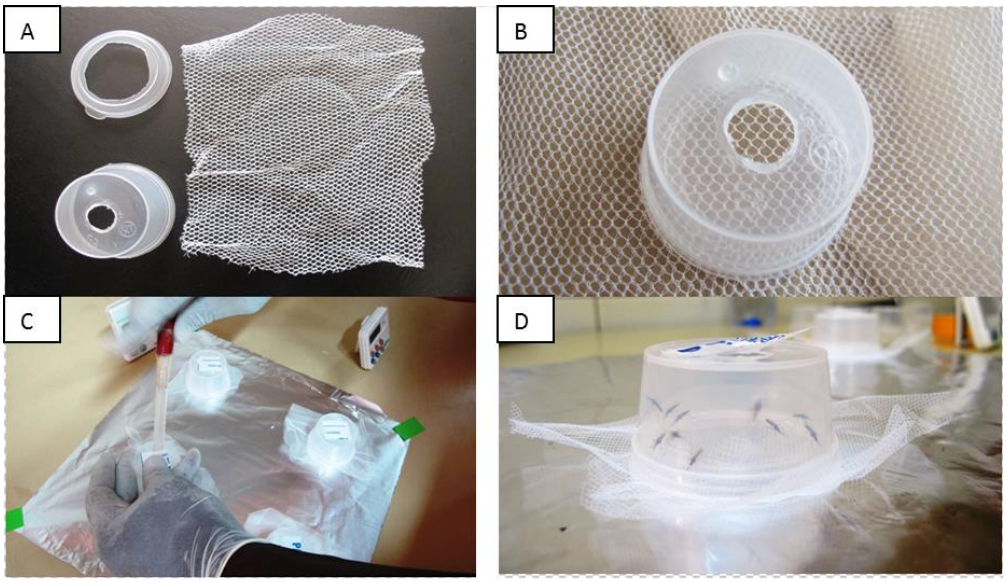


Figure 2.2 Deli pot bioassay. A) Components needed for the bioassay; B) Deli pot assembled and ready for the bioassay; C) Mosquito introduction inside

the plastic pot, using a standard aspirator and a piece of Parafilm to close the orifice; D) Mosquitoes in contact with the target net.

Table 2.3 Sample size of mosquitoes used in the longevity and fecundity experiments. Number of female mosquitoes used in each treatment for both 'Survival and lifelong fecundity' and 'Fecundity, fertility and offspring viability experiments. In the first experiment the numbers correspond to mosquitoes that were exposed to the net and were pooled. The numbers for the second experiment are for the mosquitoes that were exposed to the nets, bloodfed and survived up to 5 days.

Treatment	Survival and lifelong fecundity	Fecundity, fertility and offspring viability
-24h control	187	41
-24h PPF	167	19
-6h control	162	42
-6h PPF	171	26
IB control	126	46
IB PPF	158	36
TTN control	112	54
TTN PPF	91	48
+24h control	98	59
+24h PPF	100	45
NBM control	152	NA
NBM PPF	160	NA

2.2.2.2 Bloodmeals

A BM was offered to every cage every week until all mosquitoes died. The number of engorged mosquitoes was recorded after each BM. Mosquitoes that did not bloodfed were retained in the cage. Sucrose soaked cotton was removed at least 6 hours prior to BM in order to stimulate feeding. An insectary rabbit was immobilized using a wooden device built in CNFRP's bioterium, and then its belly was carefully shaven with a razor. After that, the animal was placed over the cage with the belly exposed to the mosquitoes. All lights in the room were turned off, and all personnel left the place for 20 minutes. Each rabbit was used to feed a maximum of four cages per day. To avoid any

possibility of contamination, different animals were used to feed treatments and control groups.

To expose the mosquitoes to PPF during the blood meal (TTN) a 15x15 cm piece of the mosquito cage's top was replaced by a piece of PPF net. Only one rabbit was used for this treatment, and after every BM session its belly was carefully washed with soap. Control mosquitoes were fed through the mesh of non-contaminated cages. In this treatment arm the length of exposure to the net was unknown, because mosquitoes were left to feed freely while being exposed to the PPF/control net during 20 minutes, rather than the 3 minutes exposure used for the other treatment arms. This was done to give the mosquitoes enough time to complete the BM.

2.2.2.3 Mortality and lifelong oviposition

Mortality was recorded daily and dead mosquitoes were removed from the cages. Two days after each BM, a plastic dish with a filter paper partially submerged in distilled water was introduced in each cage. Mosquitoes were allowed to lay eggs for three days, when the paper was retired and the eggs counted in a dissection microscope. This was done until all mosquitoes in each treatment were dead. After counting, eggs were discarded. Temperature and relative humidity were recorded daily.

2.2.3 Oviposition, hatch rate and offspring viability

In this experiment, mosquitoes exposed to PPF/untreated nets and bloodfed according to the different treatments were individually isolated in cell culture plastic tubes instead of pooling them into cages. Oviposition, hatch rate and the development of the offspring until adults were recorded. Deli pot bioassays and bloodfeeding protocols were as described above.

2.2.3.1 Oviposition and oogenesis

The definition of fecundity in the context of this study is a quantitative measure of the eggs laid by a single mosquito at a single time. Although approximately 60 mosquitoes (6 replicates) were used for each treatment, the final sample size was variable because some mosquitoes died prior to oviposition. After both

PPF exposure and BM stages were complete, mosquitoes were aspirated into flat bottomed plastic cell culture tubes (volume: 50 ml). Every tube contained a piece of filter paper over a wet piece of cotton on the bottom for oviposition, and a piece of mesh sustained by a rubber band over the top to contain the mosquito. A piece of cotton soaked in 10% sucrose solution was put on the top of the tubes for mosquito nourishment. Oviposition was recorded up to five days after BM, and mosquitoes still alive but which had not laid eggs in that period were dissected and the status of the ovaries recorded (scored as: no follicular development, abnormalities or mature eggs). Dead mosquitoes were discarded.

2.2.3.2 Hatch rate and offspring viability

Every individual egg batch was placed in separate disposable plastic pots (height 42mm, top dia. 115 mm, base dia. 85 mm) with approximately 50 ml of distilled water. A pinch of fish food was added to the pots daily after larval hatching. The total number of 2nd instar larvae produced and the number of adults emerging were recorded for every family.

2.2.4 Statistical analysis

Kaplan-Meier analysis, Log-rank tests and Cox regressions were used to assess differences in lifespan between paired comparisons of treatments and their respective control groups using the statistical software SPSS (IBM Corp., 2011). In the 'Survival' experiment, fecundity for each gonotrophic cycle was calculated as the total number of eggs/engorged mosquitoes.

For the individual mosquitoes in the 'Fecundity, fertility and offspring viability' experiment, overall egg production was calculated as the total number of eggs/total number of egg-laying mosquitoes. Hatch rate was estimated as total number of 2nd instar larvae/total number of eggs. Finally, offspring viability was calculated as the total number of adult mosquitoes/total number of 2nd instar larvae. Mann-Whitney tests were used to compare the results using SPSS (IBM Corp., 2011).

2.3 *Results*

2.3.1 Survival and lifelong oviposition

The median lifespan of mosquitoes exposed to untreated nets ranged from 7 days after the exposure took place (10 days after adult emergence) for the -6h group, to 14 days (17 after emergence) for the mosquitoes exposed and bloodfed through the net. Lifespan will be described and analysed in terms of days after emergence.

For all exposure times with the exception of -6h, mosquitoes exposed to PPF nets had a shorter lifespan than those exposed to the untreated nets (Figure 2.3). Because ages at the time of exposure and bloodfeeding changed according to each treatment, only paired comparisons (exposed to PPF vs the appropriate control) were possible. The biggest difference in terms of average lifespan between PPF-exposed and control mosquitoes was found in the TTN group where mean lifespan was 5.3 days shorter. Paired Cox regressions between PPF and untreated nets at the different PPF exposure times showed that PPF reduced longevity in all cases ($p < 0.001$), except in the -6h treatment ($p = 0.15$) (Figure 2.4). Hazard ratios (HR), a most appropriate manner of addressing the magnitude of the PPF effect on lifespan, ranged between 1.54 (95% CI 1.2 – 1.96) and 2.88 (95% CI 2.13 – 3.89) (Table 2.4).

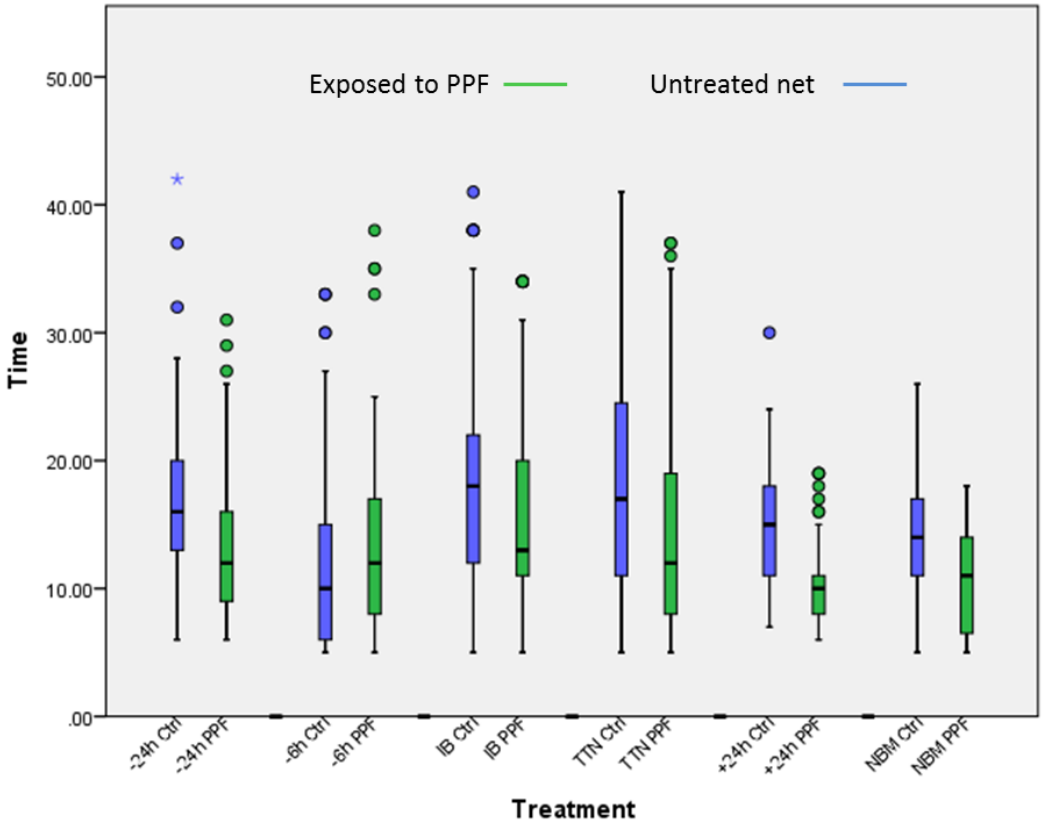


Figure 2.3 Box-and-Whisker plot showing the lifespan differences between mosquitoes exposed to PPF and untreated nets at different time points. The horizontal line inside the boxes represent the median lifespan; the lower and upper limits of the boxes represent the first and the third quartile, respectively; circles denote mosquitoes that lived beyond 1.5 times but less than 3 times the interquartile range (i.e. the size of each box), and stars denote mosquitoes that lived more than 3 times the interquartile range.

Table 2.4 Hazard ratios between pyriproxyfen exposure regimes and its negative controls. P-values and Hazard Ratios (Exp(B) in the Cox regression) show the magnitude of the effect of pyriproxyfen in mosquito mortality in the different bloodfeeding regimes. 95% confidence intervals (CI).

Treatment	p-value	Hazard ratio	Lower CI 95%	Upper CI 95%
-24h	<0.001	1.96	1.585	2.432
-6h	0.15	-	-	-
TTN	<0.001	1.795	1.342	2.4
+24h	<0.001	2.88	2.133	3.886
IB	0.001	1.536	1.204	1.96
NBM	<0.001	2.138	1.684	2.716

Feeding rate comparisons were not possible for the first gonotrophic cycle on TTN and +24h treatments because, for these cohorts, mosquitoes that did not feed were discarded. An error in recording resulted in missing data for the IB cohort. Only mosquitoes exposed to untreated nets in two regimes completed five gonotrophic cycles (Table 2.5). Although most experimental groups completed four gonotrophic cycles, sample sizes were low after the third cycle. Statistical analyses showed that among most of the times there was no difference in bloodfeeding rate between mosquitoes whether exposed to PPF or not (Table 2.5). However, all experimental regimes except +24h showed a significant difference in bloodfeeding rates at least in one of the five gonotrophic cycles. This differences were not always in the same direction: in the second gonotrophic cycle of -6h and in the fourth of IB mosquitoes exposed to PPF had a higher bloodfeeding rate than unexposed, while in the rest of significant differences it was the opposite.

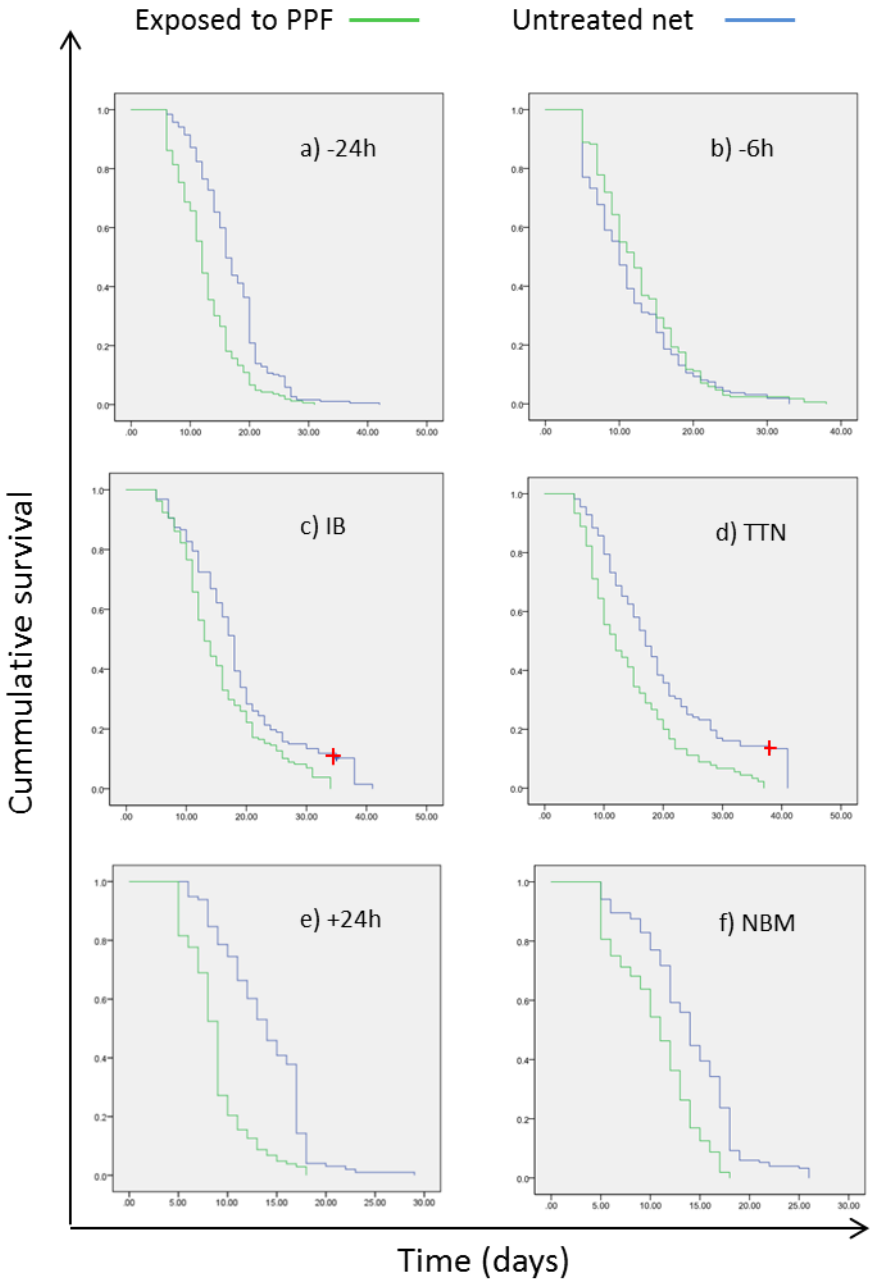


Figure 2.4 Kaplan-Meier survival curves for different bloodmeal regimes in relation to exposure to pyriproxyfen. Daily survival of mosquitoes after being exposed to a 1% PPF net or an untreated net and bloodfed at different time points (-24, -6h and IB: exposed 24 hours, 6 hours and immediately before the bloodmeal; TTN: bloodfed through the PPF/untreated net; +24h: exposed 24 hours after the bloodmeal; NBM: fed only with sucrose). The x axis corresponds to the age of the mosquitoes (days post-emergence). Red crosses (+) means censored data (5 individuals for IB control and 12 individuals for TTN control group had an undetermined age of death due to gaps in recording so the earliest possible day of death was assumed).

Table 2.5 Lifetime bloodfeeding rates. Proportion of mosquitoes visibly engorged after being offered a weekly bloodmeal. Mosquitoes were exposed to pyriproxyfen at different time points relative to the first bloodmeal (-24, -6h and IB: exposed 24 hours, 6 hours and immediately before the bloodmeal; TTN: bloodfed through the PPF/untreated net; +24h: exposed 24 hours after the bloodmeal). All mosquitoes at the beginning of TTN and +24h experiments were already fed. The p values correspond to two sample Fisher's Exact tests. Significant values ($p<0.05$) are shown in bold case.

Gonotrophic cycle	Bloodfeeding percentage									
	-24h control	**-24h PPF**	**-6h control**	**-6h PPF**	**IB control**	**IB PPF**	**TTN control**	**TTN PPF**	**+24h control**	**+24h PPF**
1st	81% (n= 184)	87% (n= 144)	63% (n= 162)	56% (n= 171)	ND	ND	100% (n= 112)	100% (n= 91)	100% (n= 98)	100% (n= 100)
	p= 0.18		p= 0.22							
2nd	88.8 (n= 143)	73.3 (n= 75)	48.7 (n= 76)	68.1 (n= 94)	92.3 (n= 104)	90.6 (n=107)	60% (n= 82)	41% (n= 50)	64% (n= 73)	44% (n= 18)
	p= 0.006		**p= 0.012**		p= 0.81		p= 0.051		p= 0.18	
3rd	30.8 (n= 39)	7.7 (n= 13)	59.3 (n= 27)	42.4 (n= 33)	86.8 (n=38)	87.2 (n=47)	82.7 (n= 52)	48.0 (n= 25)	0 (n= 14)	0 (n= 3)
	p= 0.14		p= 0.3		p= 1		**p= 0.03**		p= 1	
4th	100.0 (n= 6)	25.0 (n= 4)	57.1 (n= 7)	20.0 (n= 5)	21.7 (n=23)	91.3 (n=23)	78.6 (n= 28)	10.0 (n= 10)	0.0 (n= 1)	
	p= 0.033		p= 0.29		**p< 0.001**		**p< 0.001**			
5th	100.0 (n= 2)				6.2 (n= 16)					

n: Number of bloodfed mosquitoes

The total number of eggs laid is shown in Table 2.6. The number of eggs laid by the control groups ranged from 13.3 to 76.8 eggs/bloodfed female (Average: 33.6 eggs/bloodfed female). However it is important to note that the experimental design did not directly measure egg production per female and so the total number of females contributing to the egg output is unknown.

Mosquitoes exposed to PPF 24 hours before and after exposure (-24h and +24h) were sterilised for life (Table 2.6). In -6h and IB PPF experimental groups no eggs were laid after the first BM and a very small number were laid in subsequent gonotrophic cycles. Mosquitoes obtaining their BM through the PPF net (TTN group) laid eggs after the first two BMs but the fecundity estimates (7.2 and 4.9 eggs/mosquito) were considerably lower than the control group (Table 2.6).

Table 2.6 Number of bloodfed mosquitoes and eggs produced in each gonotrophic cycle after bloodmeals. Number of bloodfed mosquitoes and eggs produced in each gonotrophic cycle after bloodmeals. Mosquitoes were exposed to pyriproxyfen nets or untreated nets at different time points relative to the first bloodmeal (-24, -6h and IB: exposed 24 hours, 6 hours and immediately before the bloodmeal; TTN: bloodfed through the PPF/untreated net; +24h: exposed 24 hours after the bloodmeal).

	1st GC		2nd GC		3rd GC		4th GC		5th GC	
				Eggs		Eggs		Eggs		
Treatment	BFM	Eggs #	BFM	#	BFM	#	BFM	#	BFM	Eggs #
-24h control	149	6551	127	5215	12	160	7	216	3	122
-24h PPF	125	0	55	0	1	0	1	0		
-6h control	102	1645	37	1447	16	226	4	199	0	
-6h PPF	95	0	64	5	14	0	1	4	0	
IB control	ND	5241	104	3782	38	1175	23	384	16	ND
IB PPF	ND	0	107	0	47	63	23	32		
TTN control	107	4218	49	4269	43	3022	20	1525	17	1514
TTN PPF	91	654	59	287	25	0	6	0		
+24h control	98	3534	47	1888	0		0			
+24h PPF	100	0	53	0	0					

GC: Gonotrophic cycle; BFM: Bloodfed mosquitoes; ND: no data available

In conclusion, a single 3 minute exposure to PPF at different times before and after a BM significantly reduced the lifespan of mosquitoes (except when mosquitoes were exposed 6 hours before a BM). This single exposure also dramatically reduced the reproductive output of mosquitoes over multiple gonotrophic cycles.

2.3.2 Individual oviposition, hatch rate and offspring viability

In this set of experiments the treatment regimes remained the same as in the 'Survival and lifelong oviposition' experiment, but in this case oviposition and hatch rates of individual mosquitoes were recorded. Although for these experiments all mosquitoes were bloodfed, oviposition rates in the in control groups ranged from 76% (IB control) to 87.8% (-24h control) (Table 2.7). The highest fecundity estimate (number of eggs/bloodfed mosquito) in control groups was shown by mosquitoes exposed through an untreated net while obtaining a bloodmeal (TTN Control) (102.4 eggs/mosquito), while the lowest was observed in mosquitoes exposed immediately before taking a BM (IB) (58.3 eggs/mosquito). In contrast, with the exception of the TTN group, none of the mosquitoes exposed to PPF laid eggs (Table 2.7). In the TTN group, although 29.2 % of mosquitoes exposed to PPF nets laid eggs, fecundity was significantly lower than their control counterpart ($p= 0.033$).

Mosquitoes that did not lay eggs were dissected and the morphology of the ovaries examined. Ovaries were catalogued as abnormal when they were grape-like 5 days post BM as opposed to the oval-shape loose eggs expected when eggs mature (Figure 2.5). All mosquitoes from control groups, except an individual of -6h PPF control, showed ovaries with either mature eggs or undeveloped follicles. On average, the numbers of eggs observed after dissection of mosquitoes which retained their eggs were similar to the number of eggs successfully laid in mosquitoes exposed to the control group. Dissections of mosquitoes exposed to PPF showed that only the mosquitoes that obtained a BM through the PPF net (TTN PPF) and a single mosquito from the -6h group were able to develop normal eggs (Table 2.7).

Table 2.7 Individual oviposition and ovary development of mosquitoes exposed to pyriproxyfen at different times before, during and after a bloodmeal. The total number of mosquitoes laying eggs and the total number of eggs laid are shown. Oviposition was estimated as the total number of eggs/number of mosquitoes that laid eggs. Mosquitoes that did not lay eggs were dissected, and morphology of the primary follicles/eggs was assessed.

Treatment	n	Mosquitoes that laid eggs	Eggs number	Oviposition*	Mosquitoes dissected +	Abnormal ovaries	Eggs retained	Mature eggs / dissected
-24h control	41	36 (87.8%)	2827	78.5	5	0	438	87.6
-24h PPF	19	0	0	0.0	19	17	0	0
-6h control	42	34 (81%)	2980	87.6	8	1	294	73.5
-6h PPF	26	0	0	0.0	26	24	34	34
IB control	46	35 (76%)	2043	58.3	11	0	164	54.7
IB PPF	36	0	0	0.0	36	35	0	0
TTN control	54	44 (81.5%)	4505	102.4	10	0	628	78.5
TTN PPF	48	14 (29.2%)	807	57.6	34	16	647	80.9
+24h control	59	48 (81.3%)	3475	72.4	11	0	326	65.2
+24h PPF	45	0	0	0.0	45	43	0	0

*Total number of eggs/number of egg-laying mosquitoes

+Mosquitoes that, after 5 days did not lay eggs.

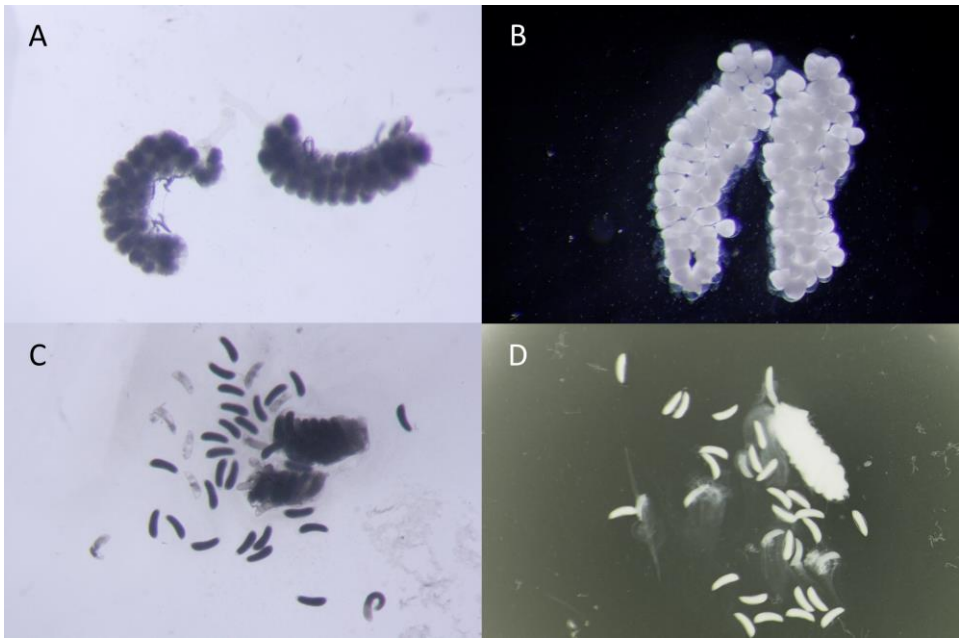


Figure 2.5 Morphology of eggs retained in ovaries. A and B) Ovaries of mosquitoes exposed to pyriproxyfen, showing round, non-detachable eggs. C and D) Ovaries with normal, oval-shaped mature eggs. Scale: 200X magnification (approximate).

The viability of the eggs laid was assessed by monitoring hatching rates and subsequent development to adult mosquitoes (Table 2.8). In the untreated net exposures, hatch rates ranged between 22.6 % (-24h (CI 95%= 13.5-31.7%) and TTN (CI 95%= 15.2-30.0%) controls) and 52.4 % (-6h control; CI 95%= 41.9-62.9%). The emergence rates in control groups ranged between 24.3 % (-24h; CI 95%= 6.0-42.9%) and 54.9 % (TTN; CI 95%= 43.8-66%).

Table 2.8 Hatch rate and offspring development. Numbers of eggs laid in every experimental group and its development until adult stage. Hatch rate was measured as the percentage of larvae that hatched and reached the 2nd instar successfully. Emergence is the percentage of adults that successfully emerged from the larvae.

Treatment	Eggs number	Larvae number	Hatch rate (%)	Adults number	Emergence (%)
-24h control	2827	638	22.6	155	24.3
-24h PPF	0				
-6h control	2980	1561	52.4	418	27
-6h PPF	0				
IB control	2043	ND	ND	ND	ND
IB PPF	0				
TTN control	4505	1018	22.6	559	54.9
TTN PPF	807	153	19	72	47
+24h control	3475	ND	ND	ND	ND
+24h PPF	0				

ND: No data available

Only mosquitoes exposed to PPF nets at the same time as blood feeding (TTN group) laid any eggs and in this group the hatch and emergence rates were similar to that in the comparable control group (Table 2.8 above).

In summary, after a 3 minute exposure to PPF before and after a BM mosquitoes were sterilised. Mosquitoes exposed to PPF while they were blood-feeding (TTN treatment) did lay eggs, although a significantly lower number than in the untreated net exposure group. These results are consistent with what was found in the 'Survival and lifelong oviposition experiment. The majority of mosquitoes that did not lay eggs when exposed to PPF showed abnormal ovaries after dissection, probably a sign of the adverse effect of PPF.

2.4 *Discussion*

This chapter reaffirmed the sterilising properties of PPF and investigated the impact of the timing of the mosquito's contact with PPF on the reductions in reproductive output and longevity.

PPF showed a clear negative effect on mosquito survival in all treatments except when mosquitoes were exposed 6 hours before the BM. It is not clear why mosquitoes from the -6h did not show a decreased lifespan compared to the control netting although it should be noted that longevity of the control mosquitoes in this experimental setting was lower than for the other experimental conditions. Mortality in -6h control group was high in the first day and increased steadily during the rest of the experiment, at a similar rate to its PPF counterpart (figure 2.5B). Factors related to the breeding of that specific mosquito batch such as larval nutrition and micro-temperature could be potential reasons for this disadvantage (Couret et al., 2014).

Ohashi *et al.* showed previously that exposure of recently bloodfed insecticide susceptible mosquitoes to PPF-treated nets decreased lifespan directly proportional to PPF concentration (Ohashi et al., 2012). However, their higher PPF concentration (0.1%) killed insecticide susceptible mosquitoes within 8 days; such a drastic effect on mortality was not observed in this study, where mosquitoes exposed to a 1% PPF net survived up to 34 days after exposure. The biological basis for the reduction in lifespan caused by PPF exposure is unknown. There are considerable knowledge gaps on the exact functions of JH and mechanisms of action, let alone the effect of its agonists on mosquitoes physiology (Wilson, 2004). It is likely that the activity of the Juvenile Hormone Esterases and other detoxification enzymes increases drastically after PPF exposure, accounting for excessive metabolic and energetic expenses and it has been shown that mosquitoes over-expressing detoxification enzymes show fitness disadvantages (Rivero et al., 2011). The fact that JH analogues are more stable and hard to metabolise than native JH (Wilson, 2004) may be negatively affecting the energetic balance in mosquitoes.

Bloodfeeding rates in mosquitoes exposed to PPF and control nets were similar but mosquitoes exposed to PPF were completely sterilised in three of the five

experimental conditions in the first study design (pooled oviposition) and four out of five conditions in the second study (individual oviposition). Mosquitoes exposed to the PPF net during blood feeding (TTN) only showed partial sterilisation. It is possible that, since the time of exposure was not controlled for this group, mosquitoes fed too quickly to acquire a sufficiently high PPF concentration to be sterilised. An alternative explanation is that the exposure was reduced as the mosquitoes legs were in direct contact with the rabbit, rather than the net as they were in other exposure regimes.

There was no apparent difference between larval hatching and adult emergence between the progeny of the TTN mosquitoes and control groups, suggesting either than PPF low concentrations are unable of affecting mosquito's offspring or simply that those mosquitoes did not get any PPF in that treatment. This finding is not consistent with Mbare *et al.*, where eggs were 13-20 times less likely to hatch into larvae (Mbare et al., 2014). Given that simultaneous feeding and PPF exposure is a likely scenario when Duo nets are in use in the field, further investigation of the impact on mosquito life histories from this exposure route is needed.

Consistent with the results, a study on *An. gambiae* mosquitoes exposed to PPF 0.1% and 0.01% impregnated nets for 3 minutes were sterilised for life (Ohashi et al., 2012). However, a concentration of 0.001% failed to completely sterilise the mosquitoes, suggesting that the impact of PPF on fecundity is likely dose-dependent. The PPF concentration used in this study was 1%, an order of magnitude higher than the highest concentration used in the mentioned study. In another study (Mbare et al., 2014), PPF (as the active ingredient of SumiLarv®) was able to reduce the proportion of egg laying mosquitoes and the fecundity and hatching rate of those which were able to lay eggs at different times before and after BM, only failing to do so when the exposure was done 2-3 days after BM. Although the duration of exposure (30 minutes) and the PPF delivery methods were different, PPF impact on reproductive traits remained similar to what is shown here. It is unknown if the lifelong sterilisation due to PPF is caused for permanent disruptions in JH-mediated gene regulation (Wilson, 2004), absence of nurse cells degeneration in follicle development (Judson and de Lumen, 1976), lack of follicle reabsorption (Judson and de

Lumen, 1976), irreversible damage of the reproductive organs (Ohashi et al., 2012) or another reason. The copulation rates were not assessed, so it was possible that some of the mosquitoes were not fertilised. Since in this study mosquitoes for both control and treatment groups came from the same batches, it was assumed that the mating rates were similar; however some males were left in the cages for delayed mating. Sexual receptivity in female insects depends on several factors, including hormonal interactions featuring prominently the JH activity (Gwadz, 1972, Barth and Lester, 1973, Ringo, 1996). There is no literature available about PPF affecting female sexual receptivity, but if this was the case it would account for a very small number of individuals in these experiments, and this would be normalised by the unexposed control mosquitoes.

Ovary dissections showed an abnormal morphology attributable to a JH analogue action in most of mosquitoes that retained eggs (Koama et al., 2015). Although there are important methodological differences between this and other recent studies done on *An. gambiae* (Harris et al., 2013, Mbare et al., 2014), in this study we confirmed that PPF sterilises mosquitoes exposed before and after a BM. Harris *et al.* reported that the only exposure to PPF time point that affected *An. arabiensis* fecundity was 24 hours after a BM (Harris et al., 2013).This timing coincides with the low physiological JH titres normal in mosquitoes at that point of ovary development. Our results differ from those findings. Differences with that and other studies are likely due to physiological processes specific to mosquito species, duration of exposure, PPF formulation, type of surface used to expose mosquitoes and timing of BM (Table 2.1) (Ohashi et al., 2012, Harris et al., 2013, Mbare et al., 2014, Koama et al., 2015).

Variation in lifelong fecundity rates between control groups could be explained by intrinsic and extrinsic reasons not necessarily related to the exposure to the untreated nets. Due to the high number of mosquitoes and replicates used in each experiment, it was not possible to do all experiments simultaneously. Lifespan and developmental rates in mosquitoes depend of several environmental factors such as temperature, larval nourishment and density (Okoye et al., 2007, Takken et al., 2013, Couret et al., 2014). Of these factors, only temperature was controlled strictly under insectary conditions of mosquito

rearing. Nevertheless this variation between control groups, the relevance of each group was in the paired comparisons with their respective PPF-exposed groups.

PPF negative effect on mosquito reproduction has been also tested in semi-field conditions. Ohba *et al.* (Ohba et al., 2013) showed that *Ae. albopictus* egg production was affected by PPF-impregnated nets. More importantly, Olyset Duo was already tested in a semi-field hut trial indicating that, additional to a higher killing rate than Olyset nets, the combination net was able to sterilize survivor mosquitoes (Ngufor et al., 2014). However, due to the low number of survivors, those results should be interpreted cautiously, and confirmation of this effect should be looked for in a proper randomised controlled trial.

In conclusion, PPF is a potent chemosterilant and an optimal candidate to integrate to a LLIN. The effect on lifespan reduction is a major addition to PPF sterilising properties, making it even more promising for malaria prevention: mosquitoes that die younger are less likely to acquire and transmit a parasite. It is unknown if these effects works with similar intensity in combination with another molecule, permethrin in the case of Duo or whether highly resistant mosquito strains can be sterilised in the same way as the susceptible. These questions are addressed in subsequent chapters.

Chapter 3 Variations in susceptibility to pyriproxyfen between populations of *Anopheles gambiae*

3.1 *Introduction*

Juvenile Hormone analogues such as methoprene and pyriproxyfen (PPF) mimic the action of the Juvenile Hormone (JH). Variation in JH levels plays a critical role in the life of insects forming part of a delicate hormonal network of interactions that are the backbone of physiological development. In holometabolous insects JH is essential for the larval development through all its stages, but its secretion must stop so the metamorphosis process and the successful emergence of adults are possible (Slama, 1971, Wilson, 2004). JH also has an essential role in vitellogenesis, which is the process of yolk formation and its uptake by the oocytes. In anautogenous mosquitoes, oocyte development goes into a 'resting stage' under the influence of JH (Gwadz and Spielman, 1973) and the fat bodies and ovaries becomes receptive to the hormone 20-hydroxyecdysone after a bloodmeal (BM) (Ma et al., 1988). However, JH synthesis stop shortly after the BM and JH-esterase activities increase, presumably to allow 20-hydroxyecdysone to function in eggs development (Shapiro et al., 1986). The presence of elevated levels of JH or JH analogues at this stage of oogenesis disrupts this process. The molecular targets, receptors and mechanisms of action of JH and JH analogues are not well known, although some advances in its characterisation have been made during the last decades (Zhu et al., 2010). JH analogues bind to the JH receptor *Methoprene-tolerant* (Met) and this binding represses metamorphosis.

Resistance to JH analogues has been reported in several species (Wilson and Fabian, 1986, Ma et al., 2010, Karatolos et al., 2012, Shah et al., 2015). In *Drosophila melanogaster* resistance has been attributed to alterations in the *Met* gene (Wilson and Fabian, 1986). Elevated levels of CYP450s were implicated in resistance in *Bemisia tabaci* (biotype B) and in an artificially selected strain of *Musca domestica* (Zhang et al., 1997, Ma et al., 2010). In the whitefly *Trialeurodes vaporariorum* PBO was able to diminish PPF resistance, and microarrays implicated the overexpression of several CYP450 genes, notably CYP4G61 (Karatolos et al., 2012).

In the Olyset Duo LLIN (Duo), the combination of permethrin and PPF aims to kill susceptible mosquitoes and sterilise the survivors. However, little is known about the potential of cross-resistance between these two chemicals. Cross-resistance between PPF and other chemical insecticides have been reported in *M. domestica* and other insects (Plapp and Vinson, 1973, Vinson and Plapp, 1974, Pospichil et al., 1996, Stara and Kocourek, 2007, Rehan and Freed, 2014) but is absent in other species (Cerf and Georghiou, 1972, Kelly et al., 1987, Keiding et al., 1991, Ishaaya et al., 2005, Cetin et al., 2009). Although not supported by a strong dataset as evidence, Braga *et al.* suggested the potential of cross-resistance between temephos and methoprene in *Ae. aegypti* (Braga et al., 2005). Cross-resistance was also described for an IGR and dieldrin in *An. gambiae* (Kadri, 1975). Plapp and Vinson (1973) suggested that a major mechanism of cross-resistance between IGR and insecticides in *M. domestica* is oxidative detoxification (Plapp and Vinson, 1973). Several mosquito CYP450s known to metabolise pyrethroids have also been shown to metabolise PPF (Karatolos et al., 2012, Nauen et al., 2015, Yunta et al., 2016). Experimental hut studies conducted in areas with pyrethroid resistant *An. gambiae* found that Duo did not reduce fecundity (Koffi et al., 2015), suggesting that this LLIN may be less effective in areas with high insecticide resistance.

PPF has been used for decades as a larvicide due to its high toxicity at low concentrations and its biosafety for non-target organisms, and most recently as a complement to pyrethroids in LLINs targeting adult mosquitoes (Duo). Thus it is important to establish assays and the determination to detect the emergence of PPF resistant mosquitoes. This is a complex task because PPF causes no immediate toxicity in adults, and its effects on survival and reproduction are various and have not been well characterised. Although the main aim of this thesis was to study the effect of PPF and Olyset Duo on mosquito reproduction, tolerance to this compound could only be compared with available literature in pupal assays by using the pupacidal formulation (SumiLarv). Additionally, the characterisation of susceptibility to PPF could be more complete if the two available insecticidal endpoints, oogenesis and metamorphosis, was evaluated. The aim of this chapter was to develop a range of bioassays, using both commercial formulations of PPF and its active ingredient, and to test these on

laboratory susceptible and pyrethroid resistant strains to measure any potential cross resistance with PPF.

3.2 *Methods*

3.2.1 Mosquito strains

Kisumu strain is an *An. gambiae s.s.* colony susceptible to all insecticides, originally collected in Kisumu, Kenya in 1990 (Vulule et al., 1994). The Tiassalé population contains both *An. coluzzii* and *An. gambiae s.s.,* and was collected in Ivory Coast in 2013; this colony has been subjected to selection pressure with the pyrethroid deltamethrin at LSTM every six months. These mosquitoes are highly resistant to DDT, permethrin, deltamethrin, bendiocarb, dieldrin, and are partially susceptible to fenitrothion (96% mortality) (pers. comm. Liverpool Insecticide Testing Establishment). Cone bioassays show that the strain can survive exposure to Permanet 2 (deltamethrin 55 mg/m^2) and Olyset (permethrin 2%) LLINs (Bagi et al., 2015). Over-expression of CYP450s and target site resistance (ACE-1 and kdr) have been reported in this strain (Edi et al., 2014). Naniagara mosquitoes were collected in larval breeding sites in Southwestern Burkina Faso as part of this study; they are highly resistant to permethrin (see Chapter 4). The F0 was used for the Duo survival experiments in this chapter.

3.2.2 Long lasting insecticidal nets

The pyriproxyfen-treated nets (PPF nets) contained 1% PPF, the Olyset nets contains 2% permethrin and the Olyset Duo nets contains 1% PPF and 2% permethrin, with an improved permethrin bleeding rate compared to conventional Olyset nets (Sumitomo Chemicals, personal communication). All these nets were made of 195 denier monofilament polyethylene with a mesh size of 75 holes/in^2. All treated nets were kindly provided by the manufacturer (Sumitomo Chemicals Ltd.) and stored at 4˚C, protected from direct light.

3.2.3 Pyriproxyfen effect on the lifespan of insecticide resistant mosquitoes

Cone bioassays (Figure 3.1) were used to assess the impact of a single exposure to PPF on mosquito longevity. Tiassalé pyrethroid resistant mosquitoes were tested in LSTM laboratories using Olyset, PPF and Olyset Duo LLINs, and Naniagara field mosquitoes were tested in Banfora (Burkina Faso) insectaries using Olyset Duo nets. Briefly, the piece of net and the plastic cones (WHO) are secured between a clean acetate sheet and a white foam board in an angle of 45˚, as recommended by WHO (Figure 3.1) (World Health Organization et al., 2013). Three to five days old female mosquitoes were acclimatised for at least one hour in ten replicates of ten individuals each, according to availability. Then they were gently introduced into the cones and exposed for three minutes to the LLINs or untreated nets. Due to the high number of mosquitoes needed, these experiments were not conducted simultaneously with all LLINs but in a paired fashion comparing a treated LLIN with an untreated control. Twenty-four hours after the exposure mosquitoes were offered a BM, the unfed discarded and the engorged pooled in polyethylene buckets (85 oz) covered by a fine mesh. Mortality was recorded daily until all mosquitoes died. A BM was offered weekly by using a Hemotek artificial bloodfeeding system, and a piece of cotton moisturized with 10% sucrose was available at all times.

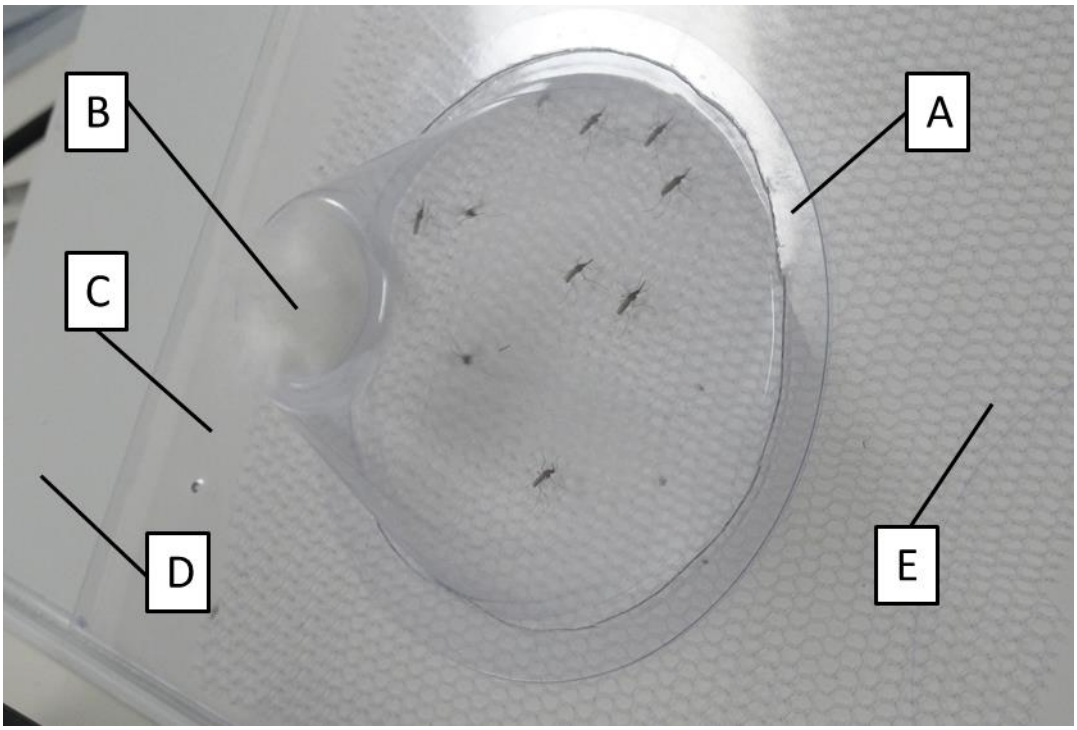


Figure 3.1 Cone bioassay setup. A) WHO plastic cone; B) Hole where mosquitoes are introduced into the cone; here plugged by a cotton piece; C) Transparent acetate sheet to hold the net and plastic cone together; D) White foam board; E) LLIN piece for testing.

3.2.4 Pyriproxyfen effect on egg development

Three to five day old Tiassalé and Kisumu mosquitoes were exposed for 3 minutes to PPF and untreated nets by cone bioassays. Twenty-four hours after the exposure, mosquitoes were offered a BM by using a Hemotek bloodfeeding system, and engorged mosquitoes were isolated in 50 ml flat bottomed cell culture tubes with a substrate for oviposition. Unfed mosquitoes were discarded. Oviposition was followed up to 5 days, and on that day survivor mosquitoes were dissected and the number of eggs in ovaries recorded. In subsequent experiments, only with Tiassalé mosquitoes, two exposure times (30 seconds and 3 minutes) were used to test the PPF, Olyset and Duo nets. Twenty-four hours after the exposure, mortality was recorded and surviving mosquitoes were bloodfed and isolated in cell culture tubes as described above. Five days after the mosquitoes were dissected and the ovary morphology recorded (i.e. oviposition was not induced).

As contact time with the netting in cone bioassays can be variable (Angela Hughes person. comm.), an alternative methodology was developed to ensure maximum contact with the treated surface. Borosilicate glass tubes (30 cm long, 11 mm wide) were coated with PPF (a.i.) at 0.55 mg/m^2, 2.75 mg/m^2 and 5.5 mg/m^2. An additional tube impregnated only with the solvent (acetone) was used as a negative control. The tubes were rotated uniformly on a flat surface to ensure an even coating, left to dry for at least 1 h and then used on the day of preparation. Two groups of fifteen 5-7 days old female mosquitoes from Tiassalé and Kisumu strains were tested for each concentration (n= 30). After acclimatisation in paper cups, they were aspirated into each tube for 3 minutes. After that, mosquitoes were returned to the paper cups and left for 24 hours with a 10% sucrose solution. Next, they were offered a BM by arm feeding, and kept for five days under insectary conditions and then dissected. The ovary morphology was then scored. Dead mosquitoes or mosquitoes not presenting egg development were discarded and removed from the analysis (*i.e.* only mosquitoes that showed egg development after dissection were considered as retaining eggs).

3.2.5 Pyriproxyfen effect on larval metamorphosis

To measure the effect of PPF on metamorphosis, SumiLarv®0.5G (Sumitomo Chemicals Ltd) was ground into a fine powder and dissolved in water to prepare a stock solution of 1000 ppm SumiLarv (50 ppm active ingredient). The solution was left overnight dissolving on a magnetic stirrer, protected from light. Serial dilutions were prepared and the following PPF concentrations were tested: 0.001 ppb, 0.005 ppb, 0.07 ppb, 0.1 ppb, 1 ppb, 5 ppb and 10 ppb. Four replicates of 25 3rd instar larvae from Tiassalé and Kisumu were exposed to each of the SumiLarv concentrations in paper cups for up to 8 days. Larvae were fed TetraMin® baby fish food every day and cups covered with netting to prevent adults escaping. The number of live and dead larvae, pupae and adults was recorded every 24 hours until all individuals were emerged as adults or dead. Adults and dead pupae were removed daily.

3.2.6 Statistical analyses

To determine the effect of PPF alone or in combination in the longevity of mosquitoes, survival Kaplan-Meier analyses and log-rank tests were done in the SPSS software (IBM Corp., 2011). Cox regressions were done to describe the magnitude of the effect of each LLIN on survival (hazard ratios) using the same software. The Dose Effect function on XLSTAT (Addinsoft) was used to estimate the concentration resulting in 50 % emergence inhibition (EI50).

3.3 *Results*

3.3.1 Effect of pyriproxyfen on the longevity of insecticide-resistant mosquitoes

The exposures to the different LLINs were not done simultaneously, therefore a specific negative control was included in each experiment. Since mortality was anticipated in the Olyset experiments and the aim was to follow the lifespan of at least 100 mosquitoes, 195 mosquitoes were initially exposed to the Olyset net; however as mortality was low (2.6%), only 108 mosquitoes (i.e. not the totality of the survivors) were offered a BM of which 97 (90%) successfully fed. In the Duo experiment with Tiassalé 238 mosquitoes were exposed and the mortality was 12.6%; from the surviving mosquitoes (208), 86% took a BM. Mortality after exposure to the PPF net was negligible, and the bloodfeeding proportion was almost 100%. Naniagara field mosquitoes exposed to Duo suffered a 38.8% mortality (n=157), and 81% of the survivors took a BM (n=96).

Bloodfed mosquitoes were pooled according to the experiment and mortality recorded daily. In each group, comparisons with the negative controls (untreated nets) were done. Cross comparisons were not done as the experiments were done at different times and, in the case of Naniagara, in a different place.

The average lifespan of Tiassalé mosquitoes was not affected by exposure to Olyset nets (average lifespan post exposure Olyset: 20.3 (CI 17.8 – 22.8) days vs negative control: 18.2 (CI 15.9 – 20.5) days) (p=0.26). However, the group of mosquitoes exposed to the PPF net showed a marked decrease in lifespan

(19.8 (CI 18.2 – 21.5) in the controls to 9.9 (CI 8.8 - 11) days for the PPF-exposed mosquitoes). The hazard ratio indicated that mosquitoes were 3.9 (CI 2.8 – 5.4) times more likely to die when exposed to the PPF net ($p<0.001$). The average lifespan of Tiassalé mosquitoes after exposure to Duo was 15.2 (CI 14.1 – 16.2) days, also significantly lower than its controls ($p<0.001$). In this case the magnitude of the negative effect on survival was significantly smaller than with PPF alone: mosquitoes exposed to Duo were 1.68 (CI 1.2 – 2.3) times more likely to die than the negative controls. A similar response was found in Naniagara wild mosquitoes, where the hazard ratio was 1.59 (CI 1.2 – 2.2). In this population control mosquitoes lived on average 16.2 days (CI 14.8 – 17.7) and exposed mosquitoes 11.4 days (CI 9.6 – 13.1) ($p=0.02$). Survival curves showing paired comparisons are showed in Figure 3.2. These comparisons only analysed delayed mortality (i.e. excluding mortality 24 hours after exposure to the insecticide). Survival analyses including the 24 h mortality showed the same results: exposures to Duo reduced mosquito lifespan (Tiassalé and Naniagara $p< 0.001$), while with Olyset nets there was no significant difference, (Olyset-Tiassalé $p=0.154$).

Weekly oviposition was also measured in each group but the number of mosquitoes bloodfeeding was not recorded, therefore it was not possible to estimate the overall egg productivity. The effect of these LLNIs on oogenesis was evaluated furtherly on individual mosquitoes in the following section.

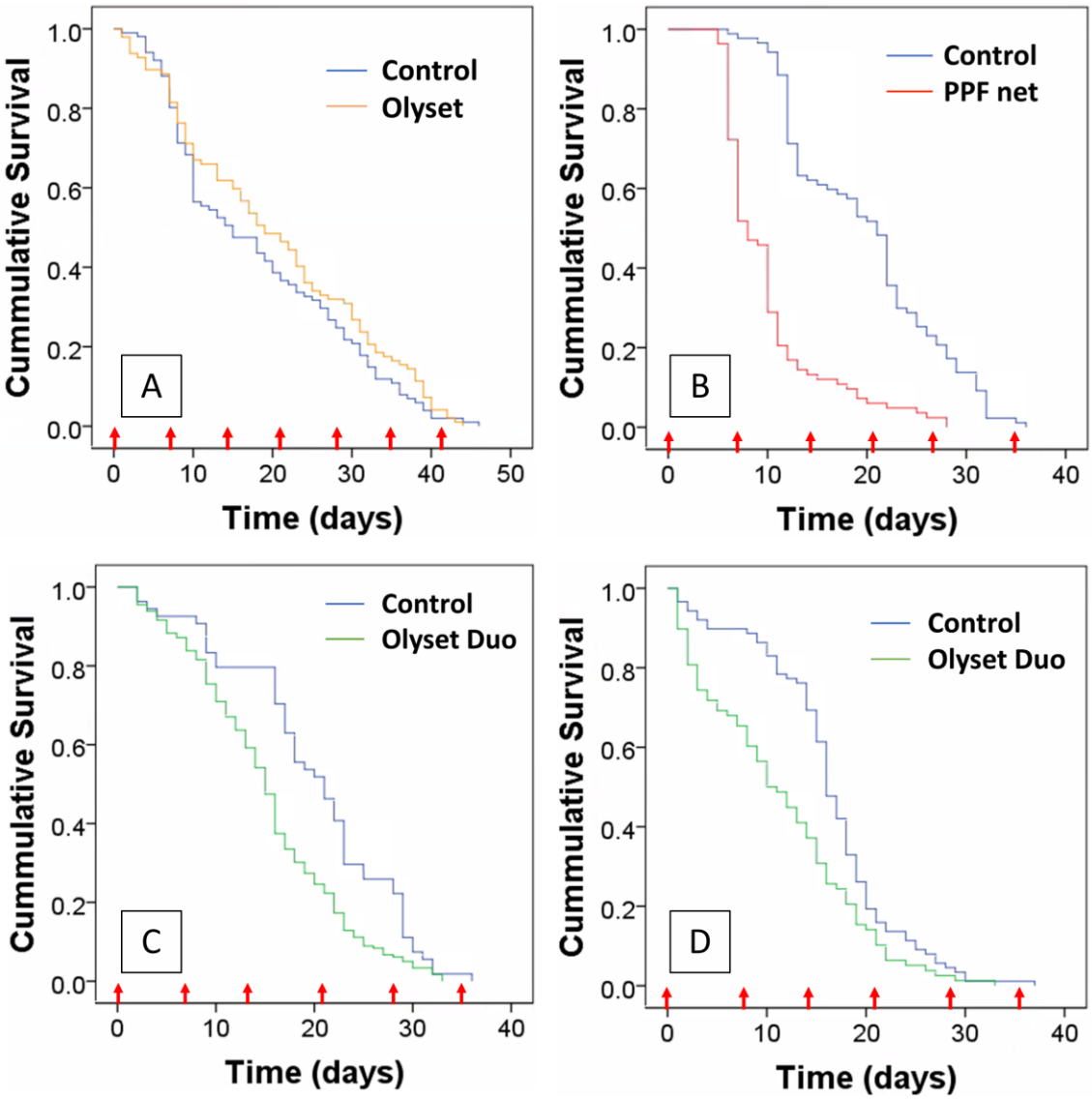


Figure 3.2 Survival curve of mosquitoes from Tiassalé and Naniagara exposed for 3 minutes to different sets of nets. All paired treated-untreated survival experiments were done at different times. Red arrows represent bloodmeals. A) Tiassalé exposed to Olyset and untreated nets (n control= 101; n Olyset: 97), B) Tiassalé exposed to pyriproxyfen and untreated nets (n control= 87; n PPF nets: 83), C) Tiassalé exposed to Olyset Duo and untreated nets (n control= 54; n Olyset Duo: 179), and D) Naniagara exposed to Olyset Duo and untreated nets (n control= 88; n Olyset Duo: 78).

3.3.2 Effect of pyriproxyfen on metamorphosis and oogenesis of insecticide-resistant and susceptible mosquitoes

In a separate set of experiments Tiassalé and Kisumu mosquitoes were exposed to untreated or PPF nets in cone bioassays and bloodfed but this time mosquitoes were allowed to oviposit individually and also dissected to assess the impact on egg development. Mortality after exposure of Tiassalé and Kisumu to the PPF net was 3.5% (n= 57) and 5.2% (n=58) respectively, and 79% and 91% of the survivors bloodfed in each group. High mortality was observed after BMs with 26.7% of the Tiassalé and 52% of the Kisumu mosquitoes dying during the 5 days of isolation prior to the dissections. None of the mosquitoes exposed to PPF laid eggs and all showed abnormal ovaries after dissections (Table 3.2). In contrast, all the control mosquitoes that survived the experiments laid eggs and had normal ovaries on dissection.

Table 3.1 Effect of pyriproxyfen (1% nets) on ovary development of insecticide resistant and a susceptible strains of *An. gambiae.* The sample size in the table corresponds with the number of mosquitoes that survived 5 days after a bloodmeal, and were able to either lay eggs or were dissected.

Mosquito strain	Net	n	Number of mosquitoes laying eggs	Average number of eggs / oviposition	Number of mosquitoes dissected	Average number of eggs / dissection
Kisumu	Untreated	39	6	58.8	33	56.3
	PPF	24	0	0	24	0
Tiassalé	Untreated	40	8	125	32	110.6
	PPF	33	0	0	33	0

After finding no apparent difference in the response of susceptible and resistant mosquito strains to the PPF net, a range of PPF concentrations were tested by the glass tube assay. The premise was that differences in susceptibility that could be masked by high concentrations could be uncovered by sub-optimal concentrations. Mosquitoes from the insecticide susceptible Kisumu strain were completely sterilized after a 3 minute exposure to 5.5 mg/m^2 whereas only 75%

of Tiassalé mosquitoes were sterilised by this dose. At half this dose, PPF had no impact on ovary development in Tiassalé but resulted in 76 % of Kisumu mosquitoes being sterilised (Figure 3.3). The proportion of Kisumu mosquitoes with abnormal ovaries was significantly higher than Tiassalé with 2.75 mg/m^2 and 5.5 mg/m^2 concentrations ($p<0.05$), but not at 0.55 mg/m^2 the lowest concentration ($p=0.24$).

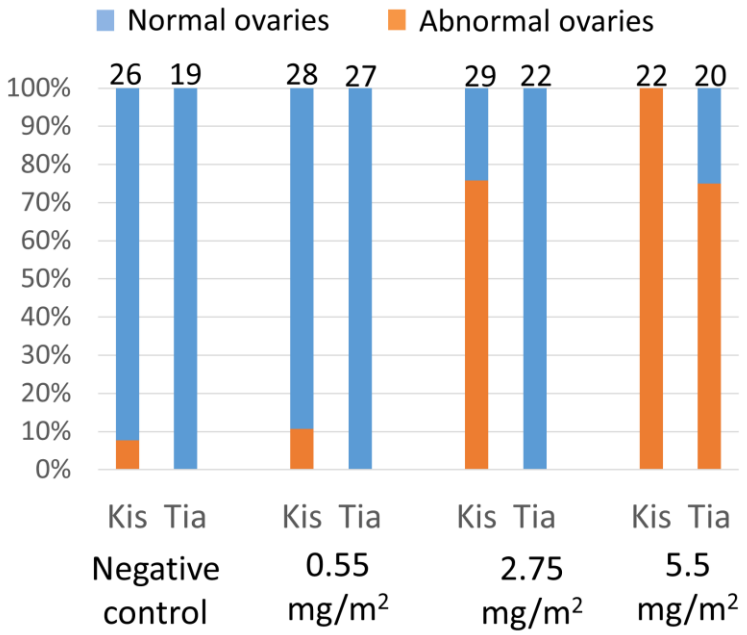


Figure 3.3 Effect of pyriproxyfen exposure on the ovary morphology of insecticide susceptible and resistant *An. gambiae s.l.* mosquito strains. Percentages of normal and abnormal ovaries in mosquitoes previously exposed for three minutes to different concentrations of PPF in a glass tube assay. The number on top of each bar represents the final number of mosquitoes dissected per treatment/strain. Under each bar the mosquito strain is stated (Kis: Kisumu, Tia: Tiassalé), as well as the concentration of PPF.

Having shown that exposure to PPF alone sterilises mosquitoes from both Kisumu and Tiassalé, the impact of simultaneous exposure to both PPF and permethrin was investigated by cone bioassays on Duo nets. Here only Tiassalé mosquitoes were used as Kisumu would be killed by permethrin exposure. Two exposure times were selected: 3 minutes and 30 seconds. Mortality 24 hours after the 30 s exposure was less than 15% for all 3 net types (2.6% for untreated, 14.3% for PPF net and 7% for Duo). After 3 min exposures to Olyset and Duo net mortality was 30.4% and 54.3% respectively (n=46 in each bioassay). The PPF mortality was equal to the untreated nets (2.2%; n=46

and n=45 respectively). PPF completely sterilised mosquitoes exposed for 3 min and sterilised 91.2% of mosquitoes exposed for 30 s. In contrast, only 60% and 15.8% of Tiassalé mosquitoes were sterilised by Duo after 3 min and 30 s exposures respectively (Table 3.3).

Table 3.2 Effect of nets with pyriproxyfen alone, absent or in combination with permethrin on ovary development after different exposure times. Exposure to each nets were done the same day and with the same batches of Tiassalé mosquitoes, and they were dissected directly 5 days after exposure instead of being induced to lay eggs.

	3 minutes		30 seconds	
LLINs	**N**	**% normal eggs**	**n**	**% normal eggs**
Untreated	23	100	39	100
Olyset	19	100		
PPF	35	0	34	8.8
Olyset Duo	10	40	38	84.2

Mosquito emergence bioassays were performed using SumiLarv, the commercial larvicide formulation of PPF from Sumitomo Chemicals Ltd. As expected, pupae mortality increased with higher concentrations (Table 3.4). The series of mortality data allowed the calibrations of the EI curves for the different strains (Figure 3.4). The SumiLarv EI50 for Tiassalé was 4 times higher than Kisumu mosquitoes.

Table 3.3 Emergence inhibition caused by different concentrations of SumiLarv in *An. gambiae* laboratory strains. Emergence Inhibition (EI%) corresponds with the percentage of mosquitoes that died before or during the process of emerging from pupae. EI50 and EI95 shows the SumiLarv (a.i.) concentration needed to cause a 50% and 95% of emergence inhibition on each strain. The numbers between brackets are the CI 95%).

	Tiassalé		**Kisumu**	
Concentration (ppm)	**n**	**EI %**	**n**	**EI %**
Control	100	1	101	14.8
1×10^{-6}	100	4	103	13.6
5×10^{-6}	100	2	102	16.7
7×10^{-5}	100	13	101	23.8
1×10^{-4}	100	19	100	41
0.001	99	69.7	100	91
0.005	102	93.1	100	100
0.01	99	99	-	-
	Emergence inhibition concentrations			
EI50 (ppm)	3.56×10^{-4} (2.7×10^{-4}-4.6×10^{-4})		8.8×10^{-5} (6.4×10^{-5}-1.2×10^{-4})	
EI95 (ppm)	0.0094 (0.0061 - 0.016)		0.0073 (0.0039 – 0.016)	

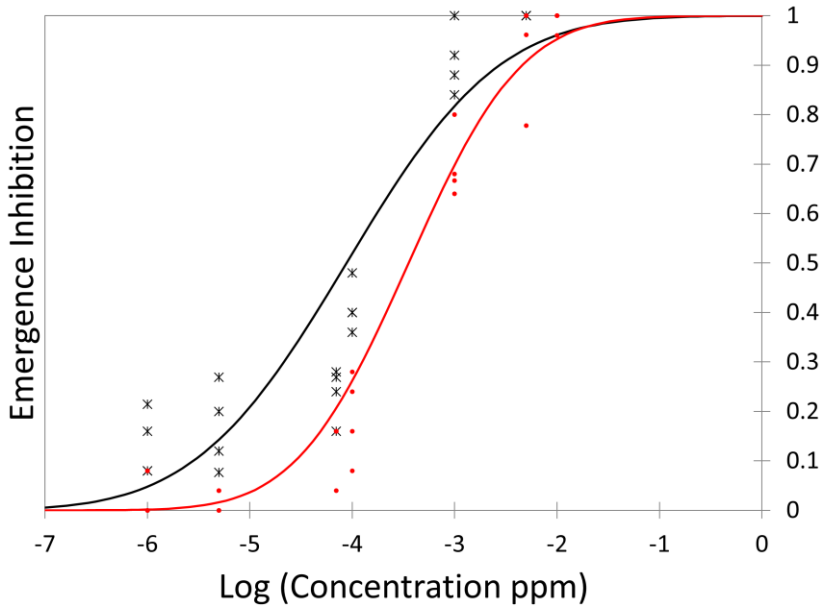


Figure 3.4 SumiLarv Emergence Inhibition 50. Emergence Inhibition (EI) curve showing the interaction between log concentration of SumiLarv and the emergence inhibition of mosquito pupae for insecticide resistant and susceptible mosquitoes. EI curves for Kisumu (black) and Tiassalé (red) mosquito strains.

3.4 *Discussion*

The experiments in this chapter aimed to detect any difference in the response towards PPF in insecticide resistant and susceptible mosquito strains using two laboratory strains. The lifespan of the insecticide resistant Tiassalé mosquitoes was reduced by exposure to the PPF nets as was previously shown with Kisumu susceptible mosquitoes in Chapter 2. The hazard ratio of Tiassalé mosquitoes exposed to the PPF nets was 3.9 (CI 2.8 – 5.4), significantly higher than the shown for Kisumu in Chapter 2 (Table 2.5) for a similar treatment (-24h): 1.96 (CI 1.6 – 2.4). Although this seems to indicate that resistant mosquitoes show a special susceptibility to the lifespan reducing effects of PPF, this differences may be due to variations in the genetic background. Further comparisons between insecticide susceptible and resistant mosquito strains will be necessary to confirm these results.

Similarly, oviposition assays after 3 minutes exposure to nets containing PPF alone did not show any difference between Kisumu and Tiassalé, with both strains being 100% sterilised. However, when the glass tube assay was used allowing mosquitoes to be exposed to a range of PPF concentrations it was

clear that Tiassalé were less affected by PPF than Kisumu with two of the three sub-optimal concentrations tested. These results highlight the importance of testing a range of concentrations in order to obtain a more detailed description of the response of susceptible and resistant strains.

One factor that was not evaluated in the oogenesis-oviposition experiments was the possibility of mosquitoes taking more than one bloodmeal before developing eggs, called pre-gravid behaviour (Gillies, 1954). The most accepted explanation for this behaviour is the need of the adult mosquito to compensate a deficient nutrition in the larval stage (Gillies, 1954, Charlwood et al., 2003). In this thesis all larvae were raised under insectary conditions with sufficient food and in non-crowded larval densities adequate for a normal development, so the possibility of pre-gravid behaviour was minimised.

Further evidence for a differential effect of PPF on Tiassalé and Kisumu is provided by the larval bioassays. The EI50 dose for Tiassalé larvae was 4 times higher than Kisumu. These differences may be due to different genetic backgrounds of the mosquito strains. However the EI50 for Tiassalé is the second highest reported so far for *Anopheles* species after *An. quadrimaculatus* with a EI50 of $1.3x10^{-3}$ ppm (Estrada and Mulla, 1986). Other studies in *Anopheles sp.* showed EI50 values between $1.7x10^{-6}$ ppm (*An. farauti*) and $1.3x10^{-4}$ ppm (*An. gambiae*) (Iwanaga and Kanda, 1988, Kawada et al., 1993, Mbare et al., 2013), within the range of what we report for the Kisumu strain. Although is true that there is no evidence in literature of cross-resistance between PPF and insecticides in mosquitoes, resistance is continuously evolving in mechanisms and strength, so this cannot be discarded and should be studied further. Despite the higher EI50 of Tiassalé, the recommended SumiLarv operational dose (0.01 ppm or 0.1 in polluted water) is still high enough to cause >99% of emergence inhibition under field settings and thus this level of resistance is unlikely to affect field performance of the product.

Duo also reduced the lifespan of field and laboratory resistant mosquitoes. A recent study showed that lifespan is also reduced in Tiassalé mosquitoes exposed to Permanet 2.0 LLINs containing deltamethrin (Viana et al., 2016). PPF-only nets increased the mortality odds of mosquitoes more than twice than Duo (Hazard ratios: 3.9 and 1.7 respectively), indicating a superior delayed

mortality effect. An experimental hut trial showed a bigger delayed mortality effect of PPF nets over Duo up to three days after mosquito catches (Kawada et al., 2014); however, this is the first time full longevity assays describe the delayed mortality effect of these nets on insecticide resistant mosquitoes. Additionally, when egg production was used as the end point to measure oogenesis, nets containing both active ingredients (Duo) were less effective than those containing PPF alone. It is unknown if the presence of permethrin is somehow antagonising the activity of PPF. Recent experiments show that several CYP450 enzymes involved in insecticide resistance metabolise and bind effectively both PPF and permethrin (Yunta et al., 2016), suggesting a competitive interaction that could affect the performance of both molecules when they are applied simultaneously. In this publication it is suggested that permethrin activity is boosted by PPF whereas PPF activity is reduced by permethrin suggesting asymmetric synergism. Experimental hut data support this by showing higher mortality caused by Duo and Olyset nets (Ngufor et al., 2014, Koffi et al., 2015). A confounding factor is that although these nets both have the same permethrin concentration, Duo has a faster permethrin bleed rate than Olyset (John Lucas pers. comm.), theoretically leading to a higher insecticide uptake by contact.

The potential cross resistance between pyrethroids and PPF is a major concern for the future of PPF, and in particular Olyset Duo, for malaria control; one of the main requirements of alternative compounds for insecticide resistance management is that no cross-resistance tolerance mechanisms should be developed in the target organism. The development and standardisation of reproducible protocols to test the effects of PPF on metamorphosis, oogenesis and longevity is of high advantage for future studies and monitoring the effectiveness in the field or in the laboratory. The sub-optimal PPF (a.i) concentrations calibrated in this study provide the first baseline available for testing PPF sterilisation effect on mosquitoes.

Mosquito tracking experiments have shown that host-seeking females spend between 17.5 – 95.6 s of contact with a LLIN in a 60 min period (Parker et al., 2015), considerably shorter than the 3 minutes used in standard WHO bioassays. The results presented here showed that 30 s exposure to the PPF

net are sufficient to sterilise >90% of the mosquitoes, whilst only approximately 15 % mosquitoes are sterilised after a short exposure to Duo. This is concerning because it is believed that one of the main drivers of insecticide resistance is suboptimal exposures, and if Duo LLINs are approved for the use in the field the development of resistance mechanisms to PPF could accelerate. Field results on the use of Duo and its implications are discussed in Chapter 5.

PPF nets having a bigger impact on mosquito lifespan and oogenesis than the combination with permethrin could be an opportunity to investigate alternatives in the design of LLINs. From a practical point of view it would be interesting to evaluate a LLIN that instead of mixing PPF and permethrin in all the panels, would have PPF-only on the roof and PPF plus permethrin in the side panels. Behaviour experiments on the interaction of *An. gambiae* with LLINs show that most of the mosquito activity is done on the roof (Parker et al., 2015). This approach is already used in Permanet 3 nets, that adds PBO and a higher concentration of deltamethrin in the roof panel (Tungu et al., 2010). It would be interesting to see if under this approach the strong impact on mosquito longevity and oogenesis shows any variation in relation to the PPF or Duo LLNIs.

This chapter provided evidence of increased PPF tolerance in a multi-resistant mosquito strain when compared with a susceptible strain, at different end points: metamorphosis and oogenesis. The inclusion of more than one insecticide susceptible and resistant strain in simultaneous experiments would reduce the genetic background confounding factors, improving the reliability of the results; this should be addressed in future studies. Further pyrethroid resistant populations with well characterised mechanisms of resistance should be assessed before cross resistance can be implicated, but the findings emphasise the importance of monitoring for PPF resistance in the field. The bioassays described within could be adapted to produce standardised methodologies to monitor for PPF susceptibility.

Chapter 4 Dynamics and mechanisms of pyrethroid resistance in *Anopheles gambiae s.l.* mosquito populations from the Banfora district, Burkina Faso

4.1 *Introduction*

In Burkina Faso, resistance has been reported in *An. gambiae s.l*. to almost all insecticides available for malaria control. A comprehensive study in 2010 revealed variability in resistance status across the country, with resistance to deltamethrin, permethrin and DDT widespread (Namountougou et al., 2012). Carbamate resistance was also reported for other sites during this period (Badolo et al., 2012, Toe et al., 2014, Toe et al., 2015). Intensity assays revealed exceptionally high levels of deltamethrin resistance in Vallee du Kou, southwestern Burkina Faso and found resistance levels, compared to a lab susceptible strain, increasing from 730 to >1000 –fold within a single year between 2012 and 2013. High permethrin resistance was also found in Tiefora Centre village, in the same region (Bagi et al., 2015). The intensity of pyrethroid resistance in Burkina Faso is affecting the effectiveness of the LLINs on malaria vectors.

The resistance mechanisms reported so far in *An. gambiae* from Burkina Faso include target site mutations (kdr and Ace-1) and increased activity of detoxifying enzymes. Although the West African kdr mutation allelic frequency has been increasing since it was first detected in Burkina mosquito populations in 2000 (Dabire et al., 2012), it is believed that its contribution to the resistance phenotype is minor compared to detoxification enzymes (Toe et al., 2015). The East African kdr mutation (L1014S) was reported for the first time in Burkinabe mosquitoes in 2013 (Namountougou et al., 2013), but no update on its frequency dynamics has been made. Transcriptional analyses over time also revealed that the expression levels of several detoxification genes increased in parallel with increases in the strength of pyrethroid resistance (Toe et al., 2015). These genes included two CYP450 genes, CYP6P3 and CYP6Z2, widely associated with resistance in other populations (Toe et al., 2015).

In Banfora district in the southwest of the country, the focus of the current study, there is intensive agricultural use of insecticides by local farmers associated

with cotton, cereals (especially rice and maize) and sugar cane (Dabire et al., 2012). This likely increases the selection pressure on mosquito populations that breed in the same area.

In June 2014 a Randomised Controlled Trial (RCT) of Olyset Duo (Duo), a bednet with chemosterilising properties containing pyriproxyfen (PPF) and permethrin, was initiated in Banfora district (Tiono et al., 2015). In addition to providing improved protection from malaria, the wide-scale use of Duo may reduce the spread of resistance by sterilising pyrethroid resistant mosquitoes that survive exposure to the net (Ohashi et al., 2012). This chapter describes the baseline characterisation of pyrethroid resistance in the study site and the results of longitudinal monitoring of resistance during the Duo trial.

4.2 *Methods*

The fieldwork was done in coordination with the Centre National de Recherche et de Formation sur le Paludisme (CNRFP) and the Banfora regional hospital.

4.2.1 Mosquito collections

4.2.1.1 Mosquito collections for susceptibility bioassays, species identification and kdr screening

Larval collections were done in several breeding sites per village as available, including semi-permanent and temporary water bodies between June and October of 2013 - 2015. *Anopheles* larvae of all stages were collected using hand dippers, and then transported to the insectaries in Banfora where they were fed with TetraMin Baby® and kept at a temperature of 27°C (±2°C) and a relative humidity of 80% (±10%). The location of the sentinel sites is shown in Figure 4.1 and the geographic coordinates are provided as an appendix (Table A1). These mosquitoes were used for susceptibility bioassays and subsequently stored in silica gel for identification of species and characterisation of *kdr* alleles.

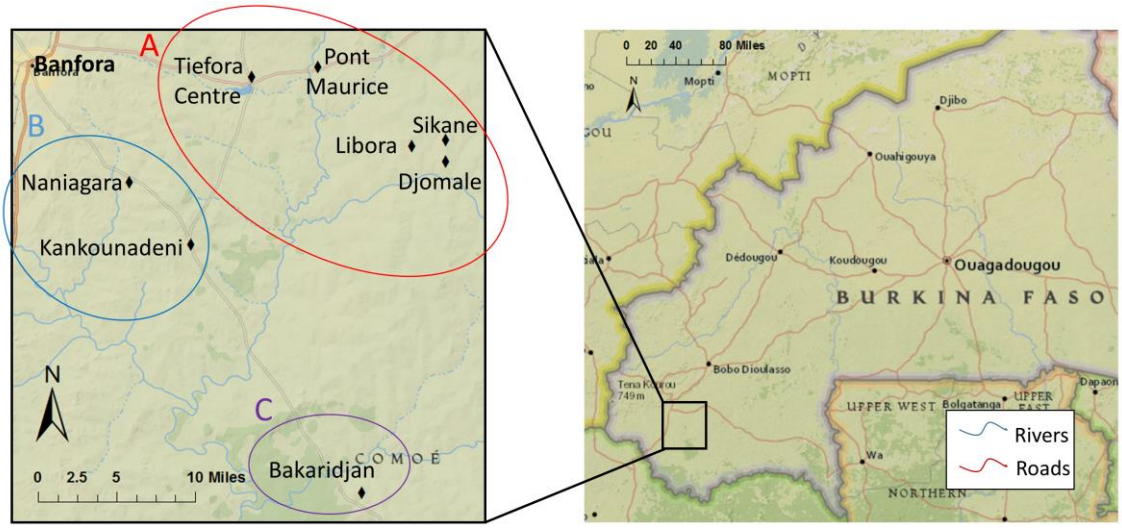


Figure 4.1 Location of the sentinel sites. Location of the sentinel sites in the Banfora district, Southwest Burkina Faso. Circles represents the villages belonging to each Health centre as follows: A: Tiefora Health Centre; B: Kankounadeni Health Centre, and C: Koflande Health Centre

4.2.1.2 Mosquitoes for microarrays

Mosquitoes for microarray experiments were obtained from different sources. Ti2013 mosquitoes were collected as larvae in 2013 from Tiefora Centre village. Larvae were raised in the local insectaries and 3-5 day old unexposed females preserved in RNA*later*®. BanS and BanM are respectively *An. gambiae s.s.* and *An. coluzzii* mosquito colonies established at LSTM, and originally collected in various sites of the Banfora district of Burkina Faso in 2014 (were Tiefora Centre village is also located). Kisumu strain is an insecticide susceptible *An. gambiae s.s.* colony native from Kisumu, Kenya, kept in LSTM insectaries, and Ngousso is a *An. coluzzii* colony native from Cameroon, also susceptible to insecticides and kept in LSTM's insectaries.

4.2.2 Susceptibility bioassays and intensity of insecticide resistance

4.2.2.1 WHO susceptibility bioassays

The 2013 revised WHO guidelines for monitoring insecticide resistance in mosquitoes were followed for these bioassays (World Health Organization,

2013). Groups of 25 three to five days old female mosquitoes were acclimatised for at least 1 h before exposure to insecticides. Then, they were exposed for 60 minutes to the WHO permethrin discrimination concentration (0.75%) and immediate knockdown recorded. Mosquitoes were left for recovery for 24 h with access to sucrose (10%), and mortality was recorded. The knockdown and mortality criteria in this and all upcoming bioassays are: mosquitoes are recorded as alive if the can stand and fly in a coordinated manner; they are considered dead if they cannot fly in a coordinated way, cannot stand or are immobile (World Health Organization et al., 2013). Susceptibility results from Tiefora, Kankounadeni and Koflande health centres were analysed in the context of the distribution of Duo (before and after) using X2 tests in Excel (Microsoft).

4.2.2.2 Lethal time bioassays

These assays are a modified version of the WHO susceptibility assay. Mosquitoes are exposed to the permethrin discriminating concentration for different time durations in order to calculate the amount of time necessary to kill different percentages of a population. The Lethal Time 50 (LT50) was calculated for different mosquito populations in 2014 and 2015 using the XLStat statistical software (Addinsoft). A binary logistic regression (BLR), with mosquito mortality as the dependent variable and permethrin exposure time (60, 90 and 120 min) and presence or absence of Duo (intervention) at the time of larval collection as the explanatory categorical variables, was performed for Tiefora health centre using the SPSS (IBM) statistical software. This analysis was performed for Tiefora health centre only because it was the only location with consistent datapoints in common before and after the distribution of Duo. The interaction between ‘time’ and ‘intervention’ was also included in the model.

4.2.2.3 Lethal concentration bioassays

A modified version of the CDC bottle bioassays was used to calculate the permethrin Lethal Concentration 50 (LC50) for each sentinel site. According to the CDC guidelines (Brogdon and Chan, 2010), sets of 250 ml glass bottles were impregnated with different concentrations of permethrin diluted in acetone.

Groups of approximately twenty five 3-5 days old female mosquitoes were exposed to concentrations ranging from 5 µg/ml to 120 µg/ml for 60 minutes, and then transferred to netted paper cups with sucrose solution available in a moisturised piece of cotton. Knockdown was recorded immediately, and mortality was recorded 24 hours after. When larval collections made it possible, each concentration was tested against 100 mosquitoes (detailed bioassay results in the table A2 in the appendix). As a negative control, an additional group of 25 mosquitoes were tested against a bottle impregnated only with acetone. All mosquitoes were stored individually in holed PCR tubes, placed into sealed bags with silica gel to avoid the decomposition of the mosquitoes and ensure the stability of the DNA. The Lethal Concentrations 50 (LC50s) for 5 sites (Tiefora Centre, Kankouadeni, Bakaridjan, Naniagara and Bounouba) in 2013 and only Tiefora Centre in 2015 were calculated by a dose-response analysis done in XLStat software (Addinsoft).

4.2.2.4 WHO cone bioassays

These assays were done following the WHO guidelines (World Health Organization et al., 2013) with some modifications. Firstly, instead of selecting panels from each side of the net, as suggested by the guidelines, an ad hoc process was used to sample the pieces from each net. Secondly, ten mosquitoes were tested for each cone instead of five. Mosquitoes were exposed to the nets for 3 minutes, knockdown recorded 1 h after exposure and mortality was recorded 24 h later. Untreated nets were used as negative controls in every experiment.

The LLINs used in these experiments were: Olyset (containing permethrin 2% or 800 mg/m^2), Olyset Plus (containing permethrin 2% or 800 mg/m^2; PBO 1% or 400 mg/m^2), Olyset Duo (containing permethrin 2% or 800 mg/m^2, PPF 1% or 400 mg/m^2), Permanet 2 (coated with 55 mg/m^2 deltamethrin) and Permanet 3® (coated with 118 mg/m^2 deltamethrin on the side panels plus 180 mg/m^2 deltamethrin combined with 1.1g/ m^2 PBO in the roof panel). The effectiveness of the nets was compared with Z-tests of proportions, done in the VassarStats website for statistical computation (http://vassarstats.net/index.html).

4.2.3 Molecular assays

4.2.3.1 DNA extraction

DNA was extracted from mosquitoes collected in 2013 by the LIVAK method (Livak, 1984). Individual mosquitoes were transferred into microplate wells containing 100 µl of hot LIVAK buffer. Then, a stainless metallic bead was placed in each well, and strip caps were secured to avoid leaking. The plate was then shaken for 3 minutes at a 30 1/s frequency, and incubated for 30 minutes at 65˚C. After that, plates were spun down and the supernatant transferred to wells in a new microtiter plate. Isopropanol was added to precipitate the DNA, and subsequent washes with ethanol were done. The DNA pellet was then diluted in 30 µl of DNAse-free water and stored at 4°C.

DNA from samples collected in 2014 was extracted by submerging two legs per mosquito in 20 µl of 1:10 diluted 1X PCR buffer (Thermo Fisher Scientific) in a 1.5 ml eppendorf tube. The tubes were closed and heated at 95 °C for 30 minutes, then spun down. Samples were stored at 4°C until use.

4.2.3.2 Species identification: SINE PCR

An. gambiae s.l. mosquitoes were identified to species level following the protocol described by Santolamazza (SINE) (Santolamazza et al., 2008). The components, conditions and interpretation of the SINE PCR are described in the Table A3 in the appendix. The products of the PCR were run in a standard electrophoresis (1.5% agarose stained with ethidium bromide) and then assessed in a UV Transilluminator. The expected size of the DNA band for *An. arabiensis* was 223 bp, 249 bp for *An. gambiae s.s.* and 479 bp for *An. coluzzii* (Figure 4.2). A 100 bp molecular ladder was used to identify the size of the DNA bands.

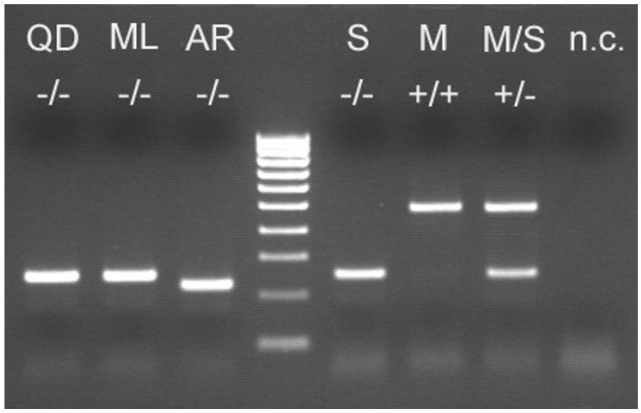


Figure 4.2 Diagnostic PCR for species identification within the *An. gambiae* complex. The results of a PCR based on an insertion in the locus *S200* X6 (represented in the image with the + and - symbols), as reported by Santolamazza *et al (*Santolamazza et al., 2008). QD= *An. quadriannulatus;* ML= *An. melas;* AR: *An. arabiensis*; S= *An. gambiae s.s.;* M= *An. coluzzii;* M/S= hybrid between *An. gambiae s.s.* and *An. coluzzii;* n.c= negative control. Figure taken from Santolamazza *et al.* (Santolamazza et al., 2008).

4.2.2.3 Kdr screening

Taqman assays were used to detect the L1014F, L1014S and N1575Y mutations (Bass et al., 2007, Jones et al., 2012). The Taqman assay consists of two probes labelled with the fluorophore FAM and VIC. The FAM probe detects the mutant allele responsible for the resistance and the VIC probe (can also be HEX or IPC) detects the wild type. The reactions were set on optical quality microplates, with 1 µl of DNA, 5 µl of Sensimix, 0.125 µl of each fluorescent probe and 3.875 µl of water per well. The temperature settings and cycle conditions were: one hold of 95°C for 10 minutes and 40 cycles of 95°C for 10 s and 60°C for 45 s. The software MxPro (Agilent Technologies) was used to analyse the results.

4.2.3.4 Microarrays

4.2.3.4.1 *RNA extraction*

RNA was extracted from pools of ten 3-5 days old female unexposed mosquitoes using the Arcturus Picopure RNA Extraction Kit® (Thermo Fisher Scientific), following the manufacturer's protocol. For the field collected samples (Ti2013) species ID was first performed on legs of each mosquito and only *An.*

gambiae s.s. were used for RNA extraction. Quality and quantity of RNA were assessed by using the Nanodrop ND-1000 (Thermo Fisher Scientific) and the Agilent 2100 Bioanalizer (Agilent Technologies). Low quality samples were discarded.

4.2.3.4.2 *cRNA labeling*

100 ng of each total RNA sample was amplified and labeled using low input Quick Amp labeling kit for 2 colours (Agilent Technologies). Each sample was labeled with Cy-3 and Cy-5 dyes in different tubes. After labeling, cRNA was purified using QIAGEN RNeasy minispin columns (QIAGEN). Labeled RNA quality and quantity were measured by the Nanodrop ND-1000 (Thermo Fisher Scientific) and the Agilent 2100 Bioanalyzer (Agilent Technologies).

4.2.3.4.3 *Hybridization*

The microarray used was the 15K Agilent AGAM_15K Chip (ArrayExpress accession number A-MEXP-2211), which contains 14071 probes for 12604 *An. gambiae* genes (Mitchell et al., 2012). Four different comparisons were made: 1) Tiefora 2013 vs Kisumu (Ti2013), 2) Banfora *An. gambiae s.s.* vs Kisumu (BanS), 3) Banfora *An. coluzzii* vs Ngousso (BanM) and 4) Banfora *An. gambiae s.s.* 2014 vs Tiefora 2013 (BanS vs Ti2013). The number of biological replicates depended on the availability of samples (Figure 4.3). Dye swapping was performed only in Ti2013 vs Kisumu.

Hybridization was performed for 17 hours at 65°C and 10 RPM. The microarray slides were then washed using the Agilent Microarray Hybridization Kit (Agilent Technologies), following manufacturers' protocol.

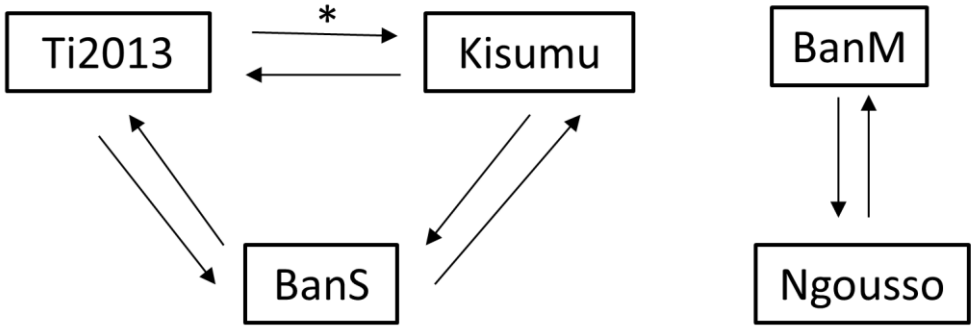


Figure 4.3 Microarray design. Scheme representing the microarray comparisons. Ti2013 vs BanS had 6 replicates, Kisumu vs BanS 5 and Ngousso vs BanM 4 replicates. *The microarrays between Ti2013 and Kisumu had dye swap, which means that the same biological samples were compared with both cy3 and cy5 dyes (i.e. two technical replicates for each of the two biological samples).

4.2.3.4.4 *Scanning and statistical analysis*

Microarrays were scanned using an Agilent G2205B microarray scanner (Agilent Technologies). The Agilent Feature Extraction software (Agilent Technologies) was employed for spot finding and signal quantification for both Cy-3 and Cy-5 dyes.

Data normalization and statistical analyses were carried out using Genespring GX software (Agilent Technologies). For statistical analysis purposes, transcripts labelled as “present” or “marginal” in 3 of 3 hybridizations were taken into account. Linear models were fit to the normalised data using the Limma package (Smyth, 2005). The selected cut-off for gene selection was of an adjusted p value < 0.01 comparing the resistant to the susceptible strain in each microarray. In the case of the temporal comparison, Tiefora 2013 against Banfora S, the cut-off adjusted p value was increased to 0.05. The reason is that since both groups were collected in the same region, relevant expression changes could be missed under more strict criteria. Finally, the three microarrays that compared the wild mosquitoes with susceptible mosquitoes (i.e. Ti2013 vs Kisumu, BanS vs Kisumu and BanM vs Ngousso) were compared with other dataset generated previously for Burkina Faso mosquito populations in Vallee du Kou and Tengrela sites at a $p< 0.05$ significant level.

In order to detect the enriched annotation terms in the microarrays, functional annotation charts were obtained by using the functional annotation tool of DAVID Bioinformatic Resources (https://david.ncifcrf.gov/home.jsp) (Huang et al., 2009).

4.3 *Results*

4.3.1 Insecticide resistance bioassays

4.3.1.1 WHO susceptible assays from 2013 - 2015

There was a high prevalence of resistance to permethrin in the study site. In 2013, 414 mosquitoes from five different villages were tested. The mortality in each site ranged from 5.5% to 33.3% with an average mortality of 20.7%; In 2014 mortality ranged from 10.1% to 19.2% in the 3 sites tested, with an overall mortality of 15.1% (n= 363); finally, in 2015 the overall mortality was 13.8%, of a total number of 702 mosquitoes tested from seven sites (Table 4.1). Mortality in 2015 ranged between 1% and 21.8% between villages.

To see if heterogeneity was reduced within smaller sampling units, two villages, Tiefora Centre and Bakaridjan, were evaluated in each of the 3 years of the study. In Tiefora Centre the percentage mortality after permethrin exposure decreased significantly during the three years (non-overlapping confidence intervals in Table 4.1), whilst in Bakaridjan resistance was more stable and did not change significantly over the duration of the study (p=0.33) (Figure 4.4, Table 4.1). Naniagara village, which was sampled only in 2014 and 2015, showed no significant change in permethrin susceptibility (Figure 4.4).

X^2 tests comparing the results of the WHO susceptibility tests before and after the distribution of Duo were done. Data from any village in Tiefora health centre (Tiefora Centre, Moussoumourou, Pont Maurice, Libora, Djomale and Sikane), Kankounadeni health centre (Naniagara) and Koflande health centre (Bakaridjan and Koflande) were used separately. There was no significant difference in mortality between mosquitoes tested before and after the distribution of Duo neither in Tiefora (p= 0.78), Kankounadeni (p= 0.08) and Koflande (p= 0.53) health centres.

Table 4.1 Prevalence of permethrin resistance over 3 years determined by standard WHO susceptibility bioassays. Prevalence of permethrin resistance over 3 years determined by standard WHO susceptibility bioassays (95% confidence intervals).

Health centre	Village	Mortality 2013	Mortality 2014	Mortality 2015
Tiefora	Tiefora Centre	33.3 % (24 – 44%)	13.4 % (7.6 – 22%)	1 % (0.05 – 6.4%)
	Moussoumourou	16.7 % (7.8 – 31%)		
	Libora			5 % (1.8 – 8.7%)
	Pont Maurice			18 % (10.5 – 28.6%)
	Djomalé			13.8 % (7.6 – 23.2%)
	Sikané			21.8 % (14.4 – 31.3%)
Koflande	Koflande	18.3 % (12.8 – 26.7)		
	Bakaridjan	21.4 % (15 – 29%)	12.3 % (14 – 26%)	16.8% (11 -24%)
Kankounadeni	Naniagara		10.1 % (5.2 – 18%)	19.8% (13 – 29%)
Madiasso	Bounouba	5.5 % (1.4 – 16%)		

In sites where more than one morphologically identical species are present, changes in species distribution may contribute to fluctuations in bioassay mortality if different species vary in their susceptibility to insecticides. Species identification was performed on the mosquitoes exposed to control papers or untreated bottles for the three villages described above and the results are shown in Figure 4.1 and in the Appendix (Table A4). During the three years of sampling, *An. coluzzii* and *An. arabiensis* were present in most of the villages in low abundance and *An. gambiae s.s.* was the predominant species (Figure 4.4). In 2013 there was a predominance of *An. coluzzii* in Tiefora (65%; n=40), but in

consecutive years this prevalence got diminished to (5% in 2014 and 2015; n= 98 and 40) (Figure 4.4). In Bakaridjan the prevalence of *An. coluzzii* was also higher in 2013 compared with the subsequent years (34%, 2.1% and 4.8% in 2013, 2014 and 2015 respectively; n= 36, 95 and 42), while in Naniagara its abundance was 10% and 8.9% in 2014 and 2015 (n= 86, 45). *An. arabiensis* prevalence ranged between 0 and 10.5% during the study (Figure 4.4).

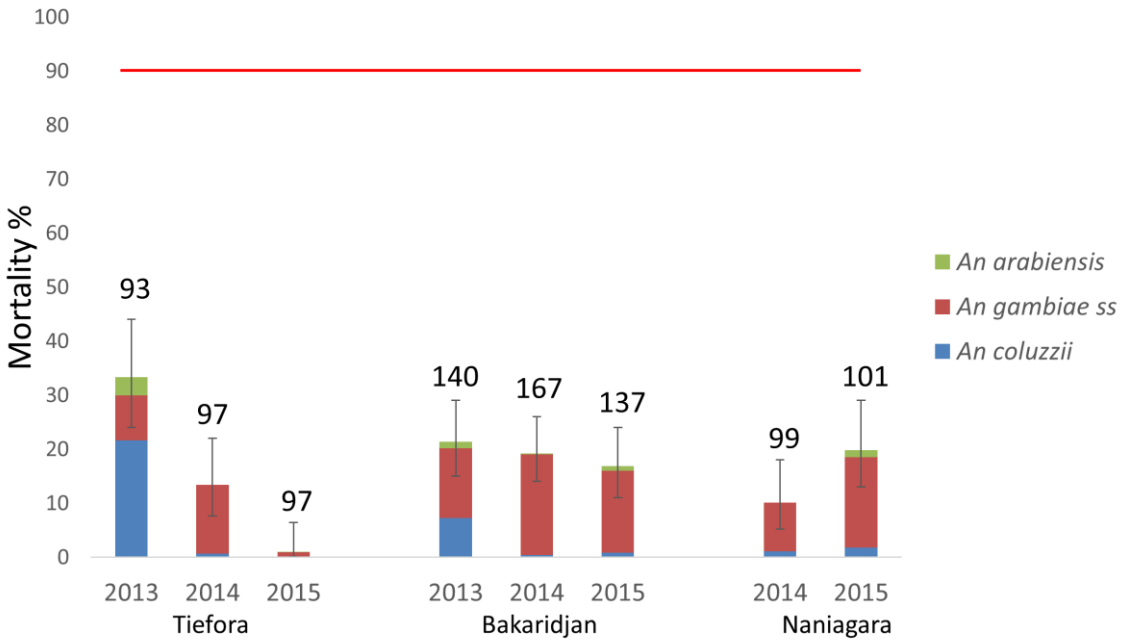


Figure 4.4 Susceptibility to permethrin in sentinel villages belonging to different health districts in Banfora followed for 2-3 years. Susceptibility to permethrin in sentinel villages belonging to different health districts in Banfora followed for 2-3 years. Numbers over each bar correspond to the number of mosquitoes tested. Different colours correspond to different species according to the relative abundance in each year (mosquitoes used were the exposed to control papers in each bioassay). The red line at 90% mortality represents the threshold established for the WHO between susceptible and resistant mosquitoes. Error bars: CI 95%.

4.3.1.2 Lethal time 50 (LT50) for 2014- 2015

Estimates of the LT50 were obtained from several sites within the boundaries of Tiefora Health Centre in 2014 and 2015. In 2014, the LT50 was calculated for a single village (Tiefora Centre) whereas in 2015 larval collections from four villages were analysed separately. The minimum sample size used for each

study site was 347 mosquitoes (average= 494). Two of the sites sampled in 2015, Pont Maurice and Sikane, generated very similar time response curves to Tiefora 2014 (Figure 4.5) with LT50s of 116 min (CI 102 - 131), 112 min (CI 101 - 123) and 97 min (CI 92 - 103) respectively; however in Libora and Djomale the estimated LT50 was approximately 2-fold higher at 232 min (CI 202 - 265) and 242 min (CI 201 - 310) respectively. The permethrin LT50 for Kisumu susceptible strain was 7.8 min, resulting in resistance ratios ranging between 12.4 times fold in Tiefora (2014) and 31.1 times fold in Djomalé (2015).

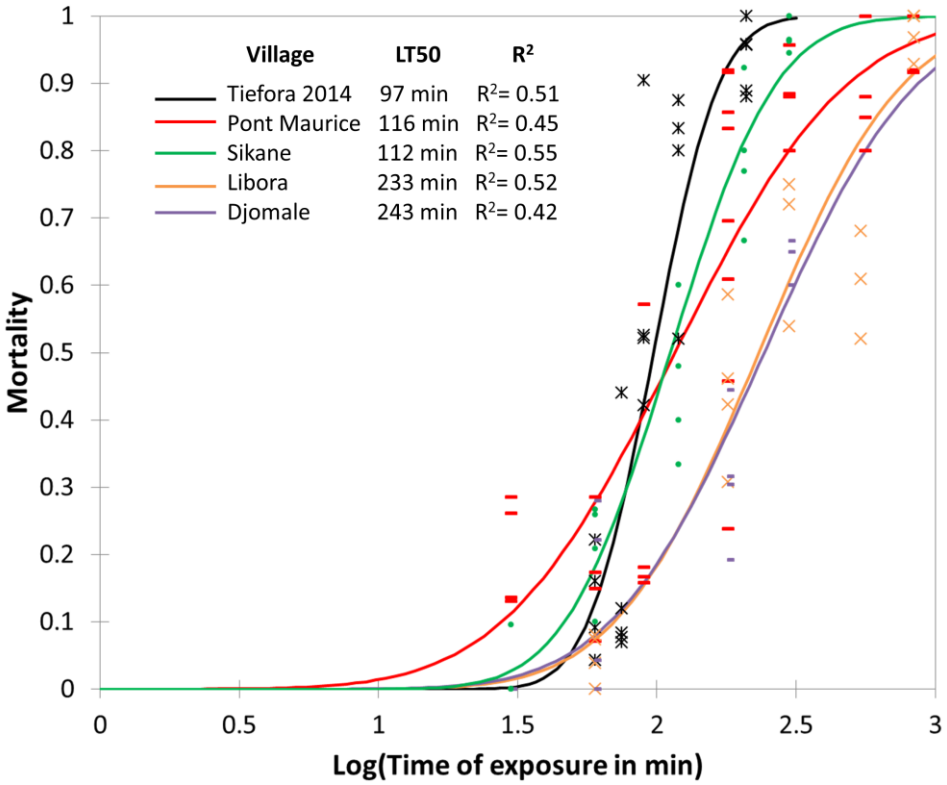


Figure 4.5 Lethal time mortality curve. Mortality curve showing 24 h mortality post-exposure to 0.75 % permethrin papers for different time periods. The Tiefora collections were performed in 2014 whereas the remaining four populations were sampled in 2015. The LT50, calculated using XLStat (Addinsoft) software is shown, along with the R^2 (Nagelkerke); all sites belong to the Tiefora Health Centre, Banfora district.

A binary logistic regression was done with Tiefora health centre data (2014 and 2015) to investigate the effect of duration of exposure to permethrin WHO papers (3 exposure times: 60, 90 and 120 min) and the intervention (distribution of Olyset Duo) on mosquito permethrin susceptibility. Exposures of 90 and 120 min were significantly different with the 60 min exposure and showed significant

influence in increasing mosquito mortality (Table 4.2). Although the intervention itself did not explain mortality (p=0.7), the interactions between the intervention (the distribution of Duo) and times of exposure (90 and 120 min) significantly showed odd ratios <1, indicating decreased odds of survival (Table 4.2).

Table 4.2 Binary logistic regression for WHO susceptibility assays with different exposure times in Tiefora health centre. Equation variables for the model explaining mortality due to 90 and 120 min exposure duration times to permethrin in comparison with 60 min, the effect of the distribution of Olyset Duo and the interactions between time exposures and the intervention.

Explanatory variables	B	S.E.	df	Significance	Odd ratios
Time			2	<0.001	
90 min	-0.614	0.297	1	0.039	0.541
120 min	-1.597	0.247	1	<0.001	0.203
Intervention					
After Duo	-0.082	0.213	1	0.702	0.921
Interactions					
Intervention * Time			2	<0.001	
After Duo by 90 min	-1.463	0.407	1	<0.001	0.231
After Duo by 120 min	-1.211	0.378	1	0.001	0.298
Constant	1.764	0.139	1	<0.001	5.836

4.3.1.3 Lethal concentration 50 (LC50) for 2013 - 2015

CDC bottle bioassays were also used to estimate the intensity of resistance in the mosquito populations of the study site. Five populations of mosquitoes were sampled in order to determine the homogeneity of permethrin resistance in the region in 2013. The number of mosquito tested per village ranged between 158 from Naniagara to 569 from Tiefora (average= 334 mosquitoes). The LC50 ranged between 17.8 ppm (CI 14.1 – 21.7) for Bakaridjan and 29.7 (CI 24 – 38.3) ppm for Naniagara (Table 4.3 and Figure 4.6). Tiefora, Bounouba and Naniagara showed a significantly higher LC50 than Naniagara and Kankounadeni (Table 4.3). The permethrin LC50 calculated for Kisumu

susceptible strain was 0.284 ppm, establishing a resistance ratio range of 62.7 to 104.6 fold times.

Table 4.3 Permethrin Lethal Concentration 50 (LC50) and resistance ratios (RR). LC50 for mosquitoes collected in 2013 in different villages of Banfora District. The RR was calculated in comparison of the permethrin LC50 for Kisumu susceptible strain, which was 0.23 ppm.

Village	LC50	95% CI		Resistance ratio
		Lower	Upper	
Tiefora	26.5	22.4	31.1	115.1
Kankounadeni	18.6	15.6	22.1	81.1
Naniagara	29.7	24.0	38.3	129.3
Bakaridjan	17.8	14.1	21.7	77.2
Bounouba	26.4	20.3	33.6	114.9

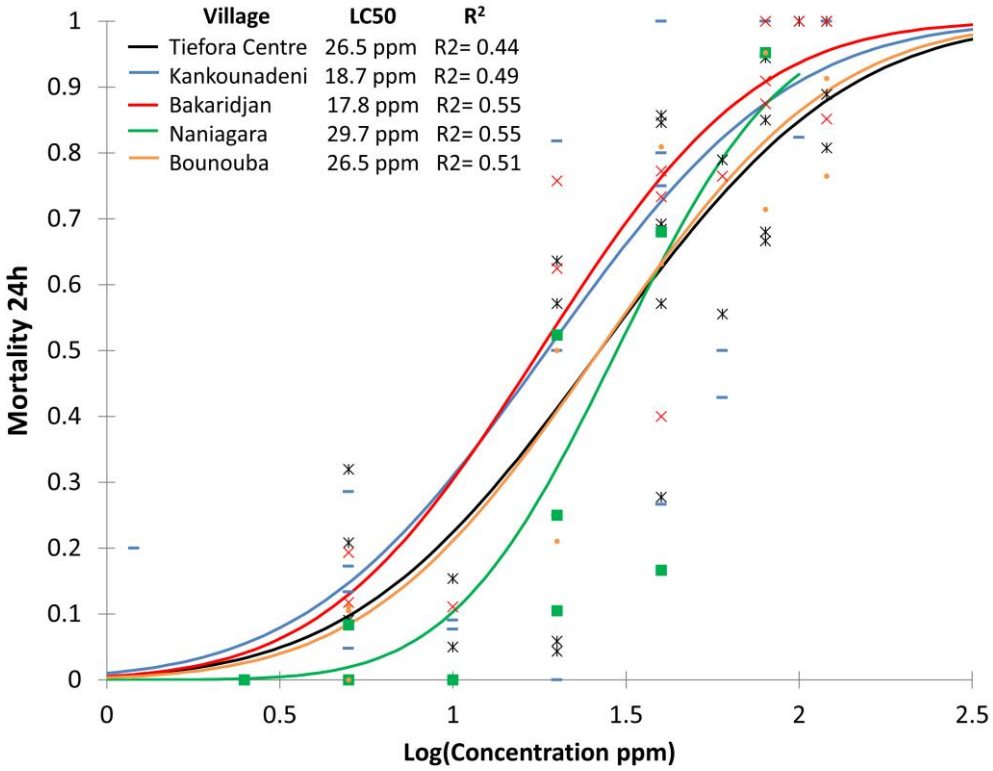


Figure 4.6 Lethal concentration mortality curve for mosquitoes collected in 2013 from Tiefora health centre, Banfora. Mortality curve showing 24 hour mortality post exposure 1 hour exposure to varying concentrations (Log) of permethrin in bottle bioassays. The Lethal Concentration 50 (LC50), calculated using XLStat software (Addinsoft), for each of the five villages.

In 2014, adults from five villages were exposed to a single permethrin dose in the bottle bioassays. This dose was selected at the permethrin LC50s calculated in the previous year for two villages (26.5 ppm for Tiefora and 29.7 ppm for Naniagara). For the remaining three villages the process was simplified to select a single dose of 20 ppm. For Tiefora and Naniagara mortality was approximately 50%, indicating a similar level of resistance to the previous years. In the remaining 3 villages, mortality after exposure to 20 ppm ranged from 55% to 65%. As the calculated LC50 for two of these villages was less than 20 ppm, the mortality greater than 50% at this concentration is not unexpected and is consistent with no major deviation in the resistance intensity between the two years (Table 4.4).

Table 4.4 Permethrin resistance in five villages from Banfora district in 2014. CDC bottle assays were performed using a single dose of insecticides with 60 minute exposure and mortality was recorded 24 hours later. The permethrin dose was selected according to the previous year LC50.

				95% CI	
Village	**Concentration**	**Sample size**	**Mortality %**	**Lower**	**Upper**
Tiefora	26.5 ppm	115	56.5	47.0	65.6
Naniagara	29.7 ppm	91	51.4	39.3	63.4
Kankounadeni	20 ppm	122	56.7	46.3	66.6
Bakaridjan	20 ppm	132	55.0	45.2	64.5
Djandoro	20 ppm	112	65.5	54.7	75.1

In 2015 a full dose response curve was generated for mosquitoes from Tiefora Centre village. In this site the resistance intensity decreased over the two years from 26.5 ppm (CI 22.4-31.1) in 2013 to 15.8 ppm (CI 14.5-17.2; n= 493) in 2015.

4.3.1.4 WHO cone bioassays

To detect the potential impact of insecticide resistance on some of the current LLINs available in the market and others in development, WHO cone bioassays were performed. All mosquitoes were obtained from larval collections, except Naniagara which were the progeny of bloodfed mosquitoes collected indoors. Very low levels of mortality were observed in all populations for Olyset nets (Table A5 (appendix); Figure 4.7 A), with mortality ranging between 1% in Bakaridjan and 10.9% in Naniagara. The addition of the synergist piperonyl butoxide (PBO) to permethrin in Olyset Plus nets did improve the toxicity of the LLINs on mosquitoes from Tiefora ($p<0.001$) and Naniagara ($p<0.05$) but the range of mortality (8.8% in Naniagara - 26.7% in Tiefora) (Figure 4.7 A) were still far below the WHO efficacy criteria. Duo performed better than the conventional Olyset in Naniagara ($p<0.0002$), but did not show any improvement against Tiefora Centre mosquitoes ($p=0.1$).

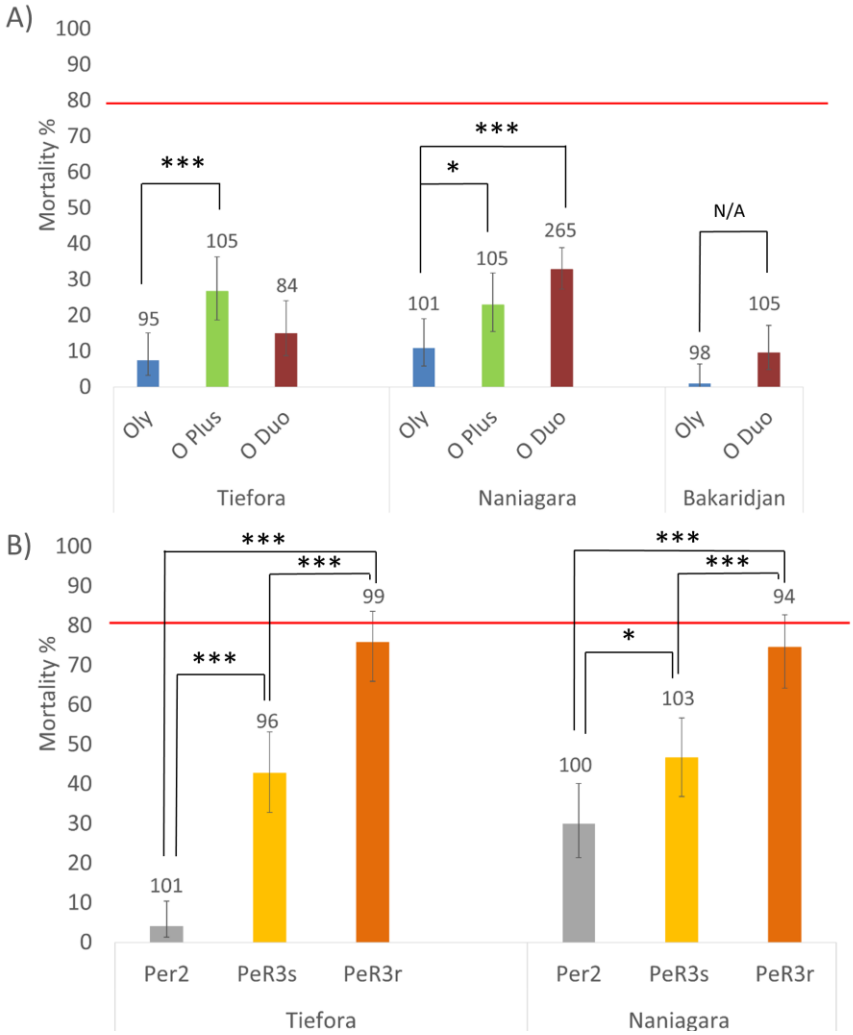


Figure 4.7 Susceptibility of mosquitoes from the study site to conventional and combination LLINs. **A)** Susceptibility of mosquito populations from the study site to Olyset (permethrin), Olyset Plus (permethrin and PBO) and Olyset Duo (permethrin and PPF); **B)** Susceptibility of mosquito populations from the study site to Permanet 2 (deltamethrin), Permanet 3 side (deltamethrin with improved bleeding rate) and Permanet 3 roof (deltamethrin and PBO). Error bars represent 95% confidence intervals. Z-tests significant differences: * = $p<0.05$, *** = $p<0.001$. The red line represents the minimum effectiveness of LLINs recommended by WHO. Numbers over each bar represent the number of mosquitoes tested per net. Abbreviations: Oly= Olyset (blue); O Plus= Olyset Plus (green); O Duo= Olyset Duo (red); Per2= Permanet 2 (grey); Per3s= Permanet 3 side (yellow); Per3r= Permanet 3 roof (orange).

Permanet nets were tested only in Naniagara and Tiefora Centre mosquitoes. For both populations, the roof panel of Permanet 3 performed better than the side panels of Permanet 3 or Permanet 2, and the side panels of Permanet 3 were more lethal than Permanet 2 ($p<0.05$) (Table A5 (appendix); Figure 4.7 B).

Although mortality by Permanet 2 was higher in Naniagara (30%) than in Tiefora (4%), mortality caused by both components of Permanet 3 showed similar results for both strains.

4.3.2 Target site resistance: kdr

During the three years of sampling the *An. gambiae s.s.* mosquitoes collected were predominately homozygous for the 1014F kdr mutation, and the allelic frequency was > 0.95 in all collections (Figure 4.8). There was no significant difference in the allelic variations between 2013 and 2015 (see Table A6 in the appendix). *An. coluzzii*, whose sample size was limited because of availability, showed a more variable kdr allelic profile: in 2013, the percentage of 1014F homozygotes ranged from 44.4% (n=18) in Tiefora Centre to 95.6 % (n=22) in Bakaridjan with allelic frequencies ranging from 0.7 to 0.95.

The 1575Y allele was not as prevalent as 1014F with allelic frequencies ranging from 0.21 to 0.43 in *An. gambiae* in 2013. This frequency did not change significantly across time in either *An. gambiae* or *An. coluzzii* (Table A6 (appendix); Figure 4.8).

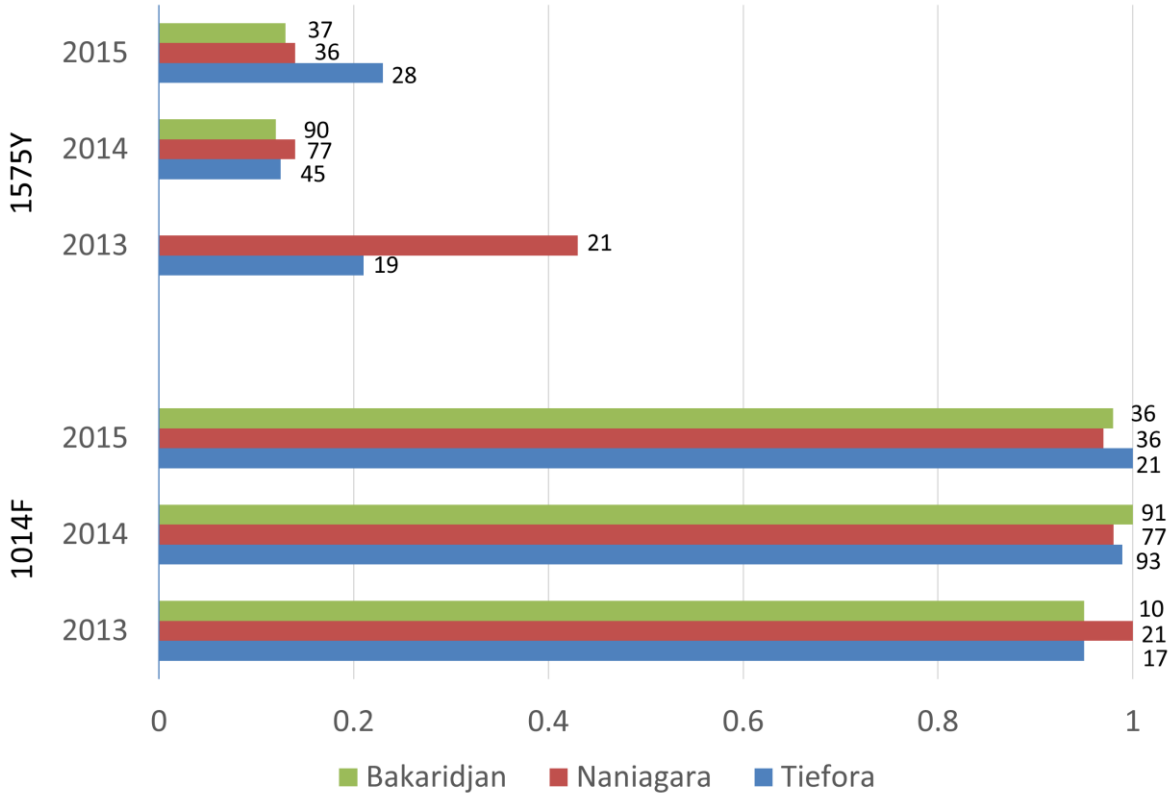


Figure 4.8 Kdr frequency in *An. gambiae s.s.* from the study site in 2013 – 2015. Allelic frequency of the 1014F and 1575Y kdr mutations for *An. gambiae s.s.* in three mosquito populations within the study site during 2013-2015.

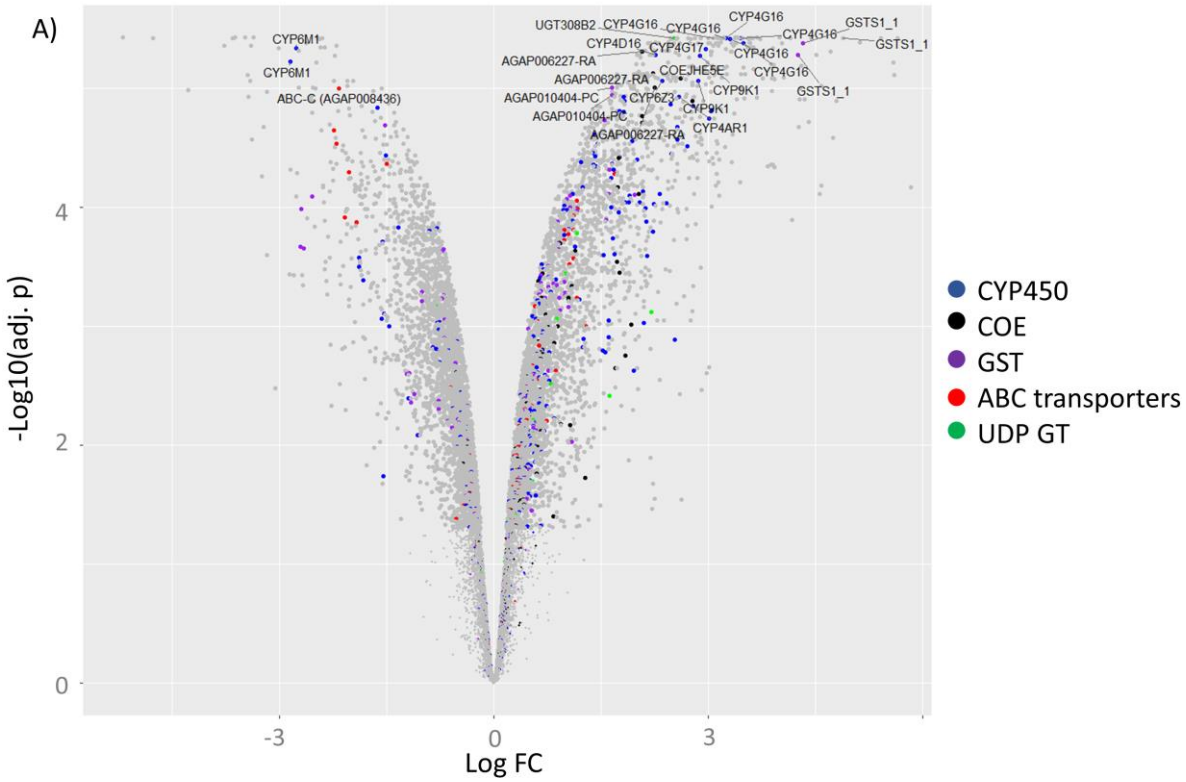
4.3.3 Microarray analysis

4.3.3.1 Comparison of the transcriptome of Banfora populations with laboratory susceptible colonies.

Pyrethroid resistance is almost ubiquitous in *An. gambiae s.l.* from West Africa making it challenging to identify changes in the transcriptome associated with resistance by comparing sympatric populations. Therefore, in the microarray experiments in this chapter, populations originating directly from field collections in Banfora (in 2013) or from colonies recently established in the laboratory from Banfora field collections in 2014 (BanM and BanS) were compared with laboratory susceptible populations. Gene expression observed cannot conclusively be linked to resistance using this approach; differential expression may simply represent variation between the strains that may be attributed to their different geographical origin, length of time in colonisation or other

unknown variables. In recognition of this limitation, four different approaches were applied to the analysis of the microarray data.

Firstly the data from each individual experiment was analysed separately to identify the most significantly over and under expressed genes in the single comparisons between resistant and susceptible populations. This data is shown in volcano plots in Figure 4.9 (Panels A-C) and the gene lists with the 20 genes with the highest fold change per microarray (upregulated and downregulated) are contained in the appendix (Table A7). The number of total upregulated genes with a cut-off significance of $p<0.01$ was 2798 for Ti2013 vs Kisumu (Ti2013), 2560 for BanS vs Kisumu (BanS) and 1376 genes for BanM vs Ngousso (BanM). The number of downregulated genes was approximately similar, with 2581 for Ti2013, 2598 for BanS and 1472 for BanM.



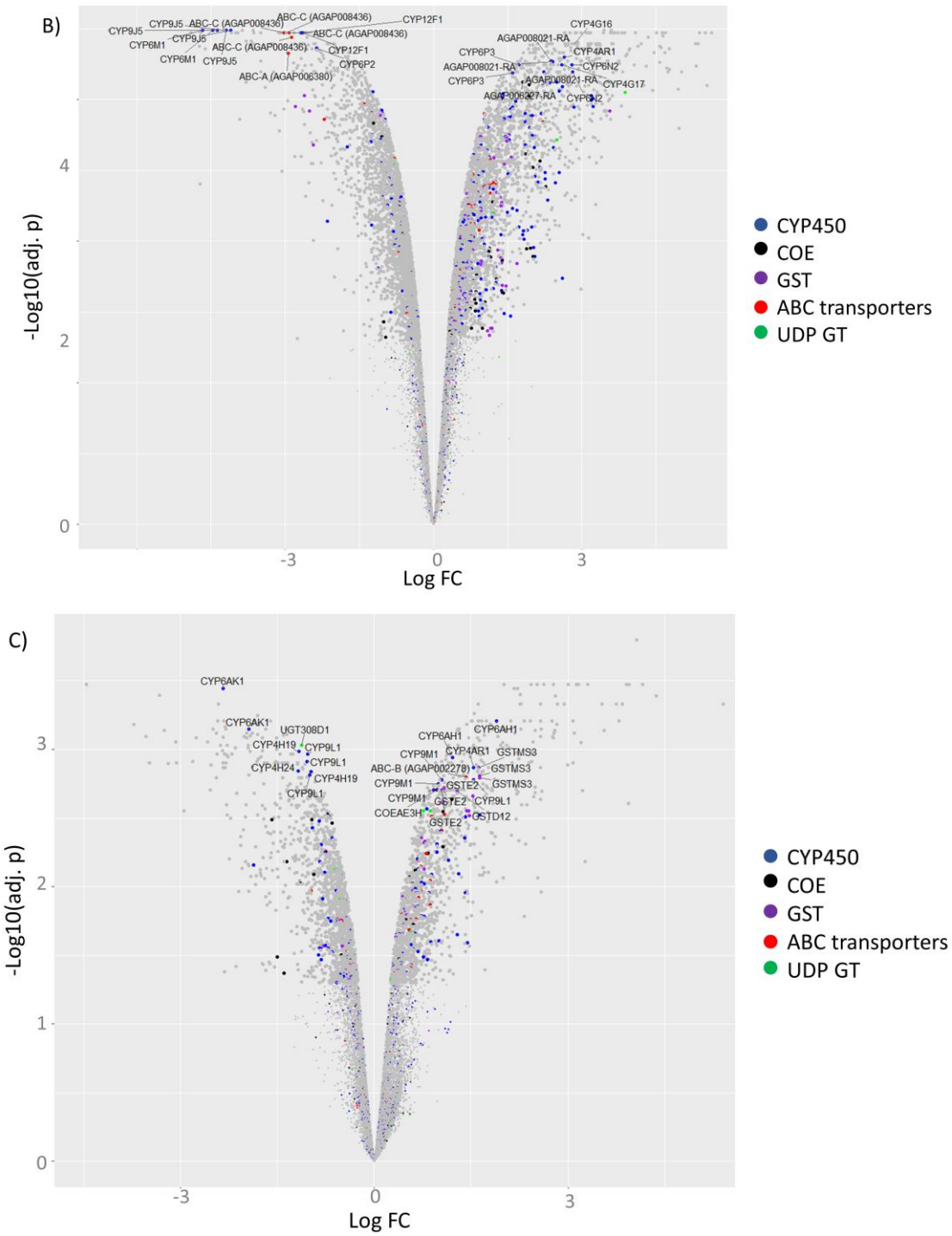


Figure 4.9 Volcano plots showing over-expressed and under-expressed probes per microarray experiment. Differential transcription is presented as the log10 of the adjusted significance contrasted with the log fold change (log FC). Transcripts elevated in the resistant populations are on the positive side of the X axis and under-transcribed in the negative side. Detoxification genes are encoded by different colours, and the size of the spot represents significance. The most significant transcripts belonging to detoxification families are named. **Panels:** A) Kisumu vs Ti2013; B) Kisumu vs BanS; C) Ngousso vs BanM.

The 20 genes overexpressed with the highest fold change (FC) in the Ti2013 microarray contained three annotated genes: the glutathione s-transferase GSTS1 gene (FC= 29.5), the cuticular gene CPR75 (FC= 27.7) and a SP11372 serine-like gene (FC= 20.4). Within the top 20 overexpressed in the BanS microarray 8 genes were shared with Ti2013 including CPR75 (FC= 31) and SP11372 (FC= 39.7) but not GSTS1 (FC= 1.6, p= 0.03); the other annotated genes included five cuticular genes: CPF3, CPCFC1, CPLCG1, CPLCG4 and CPLCG5 (Table A7). Finally, the top 20 list from the BanM microarray included three annotated genes: the LYSC7 lisozyme (FC= 46.5), the CHYM1 chymotrypsin (FC= 26) and the salivary gland gene SG2b (FC= 21.4). The most enriched annotation terms across these microarrays were: the secondary metabolites biosynthesis, transport and catabolism ranging from 42.4% to 43.8% of annotated terms; metabolism of xenobiotics by cytochrome P450 (9.8% - 11.1%); drug metabolism - cytochrome P450 (9.8% - 11.1%); glutathione metabolism (11.2% – 12.1%); lipid metabolism (7.9% – 11.1%), and peroxisome (4.5% – 5.9%) (Table A7, appendix). The analysis of the underexpressed lists of genes showed that the Secondary metabolites biosynthesis, transport, and catabolism terms were also enriched in the three microarrays (34.2% – 40%). Only in Ti2013 underexpressed genes, other categories were enriched too (Posttranslational modification, protein turnover and chaperones: 21%; glutathione metabolism: 7.9%; drug metabolism - cytochrome P450: 5.3% and metabolism of xenobiotics: 5.3%). Given the majority of enriched terms related to xenobiotic detoxification and clusters of terms related to the action of CYP450s and glutathione action, and one of the main differential traits between the mosquitoes compared in the microarray (*i.e.* insecticide resistance) a special emphasis will be done on detoxification genes.

The 20 most down regulated genes in the Ti2013 microarray included three annotated genes: CLIPB17, PGRPS3 and RpS11. These genes were also part of the top 20 down regulated genes in the BanS microarray in addition to the CEC1 and the CYP9J5 genes. Finally, the top 20 down regulated genes in the BanM microarray showed 4 annotated genes: A5R1, CHYM2, REL2 and LRIM4 (Table A8, appendix).

The proportion of upregulated detoxification genes (over the total upregulated genes) ranged from 3.9% to 8% across microarrays, and the downregulated ranged from 4.2% to 7.5%. The most differentially transcribed detoxification gene family was CYP450s, accounting for approximately 50% of all detoxification genes in each microarray (Figure A1, appendix).

Secondly we compared the transcripts differentially expressed in the same direction (i.e. up regulated in one or more resistant populations or down regulated in one or more resistant populations) between the experiments (Figure 4.10). Although the experimental design involved two different members of the *An. gambiae* species complex, genes under selection pressure have been shown to introgress between species (Clarkson et al., 2014, Norris et al., 2015) and therefore the BanM population was included in this comparison. 114 genes were overexpressed in the Ban M (*An. coluzzii*) and the two *An. gambiae s.s.* (BanS and Tie2013) populations compared to their susceptible controls and 82 were under-expressed. The over-expressed genes included 7 CYP450s (CYP4AR1, CYP4C27, CYP4D15, CYP4G16, CYP6AH1, CYP6P3 and CYP6Z2), 1 microsomal GST (GSTMS3), 1 ABC transporter (ABCB4) and 10 cuticular proteins (CPRs) (Table 4.6). The complete list of differentially expressed genes is shown in the appendix (Table A9).

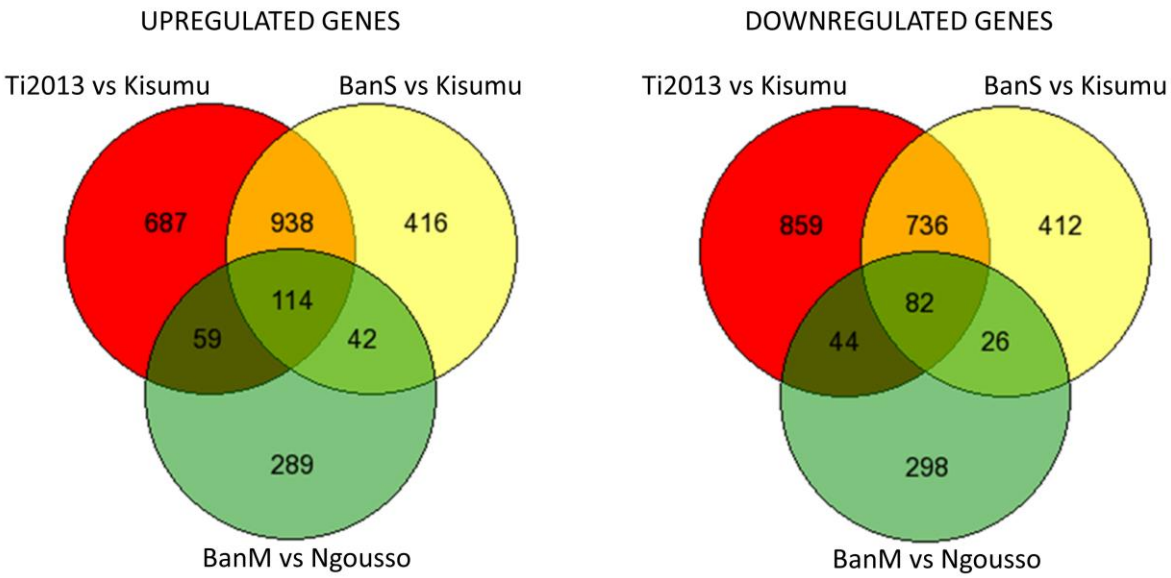


Figure 4.10 Venn diagrams showing the shared differentially transcribed genes per microarray. Genes differentially transcribed in each microarray (adjusted p< 0.01) between the resistant and susceptible mosquito populations.

Thirdly, for *An. gambiae s.s.*, field populations collected in 2013 (Ti2013) were directly compared to mosquitoes collected the following year in the same region (BanS). The 2014 collections had been maintained in colony for approximately 9 generations prior to use. 1978 genes were differentially expressed between these two populations (p< 0.05) (Figure 4.11) but only one of these (GAM1, a gambicin anti-microbial peptide) was overexpressed using a more stringent p value (p< 0.01). Interestingly 8 of the 20 genes with the highest overexpression change in 2014 compared to 2013 were cuticular genes (Table A8, appendix). Genes differentially expressed in this comparison may have arisen due to changes in selection pressure in the field between 2013 and 2014. However, it is equally plausible that the difference may be in part attributed to the impact of colonising the BanS population at LSTM. A three way comparison was therefore performed to identify genes commonly differentially expressed between the two *An. gambiae* resistant populations from the field when compared to the same susceptible population, and to identify any genes within this shared subset that were also differentially expressed between the 2013 field collections and the 2014 lab strain (BanS vs Ti2013) (Figure 4.12).

Table 4.5 Detoxification genes up and down-regulated in three different microarray experiments in mosquitoes originating from the study site. Detoxification genes overexpressed comparing field or recently colonized mosquito populations against susceptible strains, classified by family. Cytochrome P450s: CYP450s; Glutathione S-transferases: GSTs; ABC: ABC transporters. The values represent the absolute fold change. Genes marked with *, # and @ symbols were also found overexpressed in *VK6, #VK7 and @Tengrela microarrays.

Gene name	Microarray		
Upregulated	**FC Ti2013**	**FC BanS**	**FC BanM**
ABCB4	2.2	2.24	2.2
CYP4AR1*#@	6.5	5.0	3.8
CYP4C27@	3.1	5.0	2.2
CYP4D15	4.8	3.5	2.8
CYP4G16@	9.7	8.2	1.8
CYP6P3*#@	3.3	2.8	3.3
CYP6Z2*#@	3.0	2.7	3.5
GSTSM3*@	3.9	2.9	4.4
Downregulated	**FC Ti2013**	**FC BanS**	**FC BanM**
CYP325D2	0.7	0.5	0.4
CYP6M4	0.4	0.5	0.4

Over 1000 probes were over expressed in both resistant populations compared to Kisumu and 126 of these were also upregulated in the BanS 2014 population compared directly to the Tiefora 2013 population (Figure 4.12). This subset included four CYP450s (CYP4AR1, CYP6M3, CYP6Z3 and CYP9K4), two GSTs (GSTS1 and GSTMS3) and one COE (COEJHE5E). No detoxification gene was underexpressed in the shared list of genes.

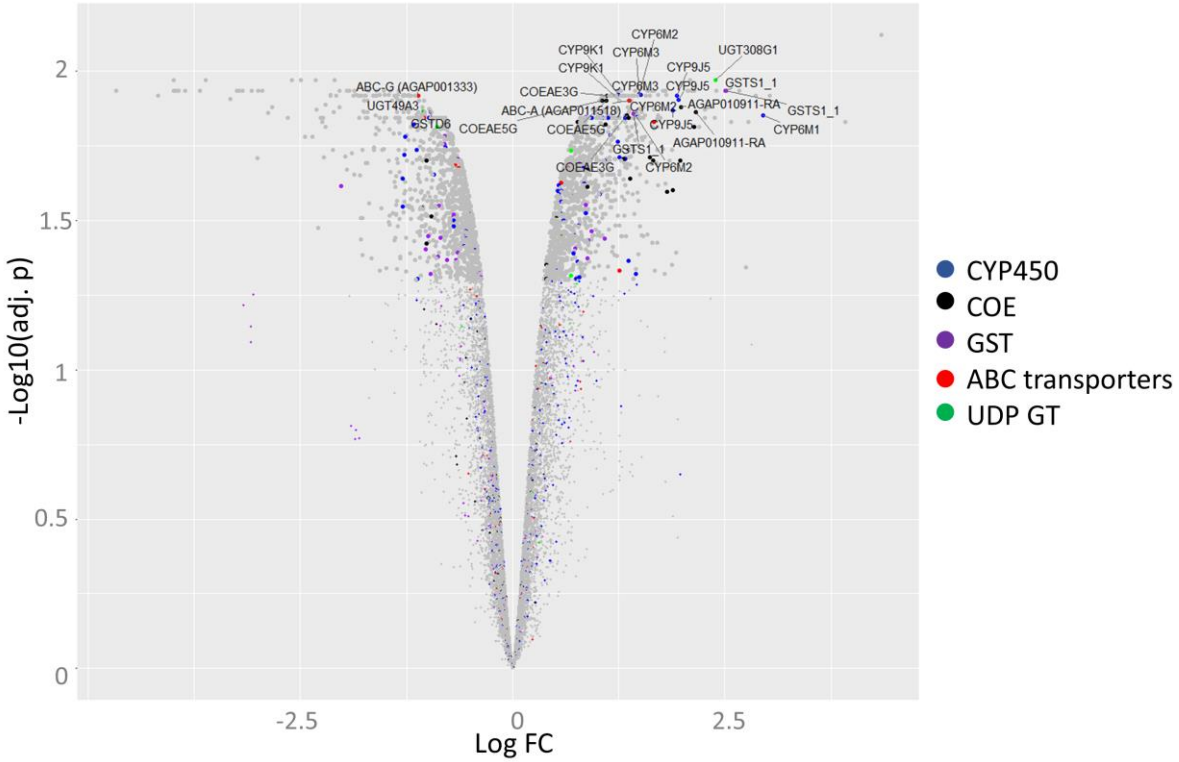


Figure 4.11 Volcano plot showing over-expressed and under-expressed transcripts in the BanS vs Ti2013 microarray. Differential transcription is presented as the log10 of the adjusted significance contrasted with the log fold change (log FC). Over-transcribed transcripts are on the positive side of the X axis and under-transcribed in the negative side. Detoxification genes are encoded by different colours, and the size of the spot represents significance. The most significant transcripts belonging to detoxification families are named.

Finally, for *An. coluzzii*, the genes lists described above were compared with other experiments from neighbouring regions or Burkina Faso with also high levels of insecticide resistance (two populations from Valle de Kou (VK6 and VK7) and Tengrela). In total, 710 transcripts were differentially expressed across all Burkina Faso populations. Detoxification genes shared between the studies are shown in Table 4.6.

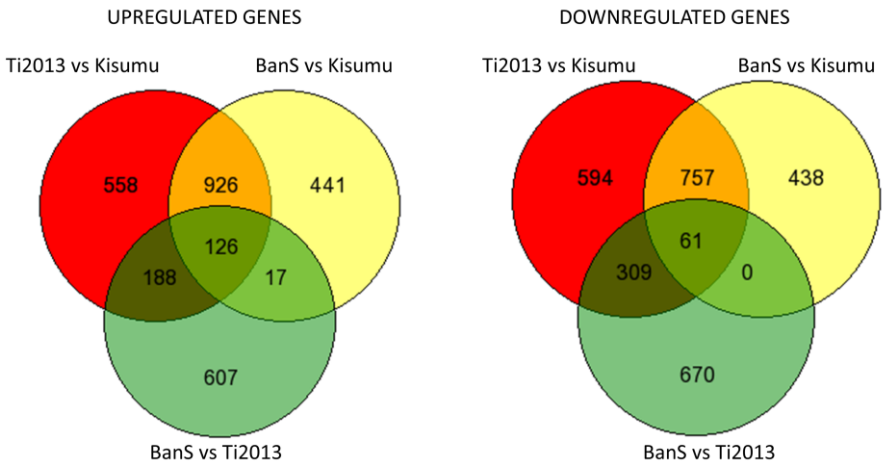


Figure 4.12 Venn diagram showing the shared statistically significant probes between Banfora mosquitoes from 2013-2014 (Ti2013-BanS) and compared with susceptible mosquitoes. This diagram shows the number of genes shared between three microarrays using the same populations/species in two consecutive years: Ti2013 and BanS separate microarrays compared with Kisumu (cut-off significance: $p<0.01$) and a microarray comparing directly BanS with Ti2013 ($p<0.05$).

4.4 *Discussion*

The results of this chapter provide data on the level of physiological resistance, the molecular mechanisms of that resistance and its impact on the efficacy of bednets, for malaria vectors belonging to the study site of a large-scale clinical trial of Olyset Duo nets in Burkina Faso (Tiono et al., 2015). The aim was to describe the characteristics of the malaria vector population in the study site and to assess the impact of the roll out of nets containing PPF on the level of pyrethroid resistance in the vector population. Results from the clinical trial itself are beyond the scope of this thesis.

4.4.1 Permethrin resistance in the study site

There was a very high prevalence of permethrin resistance in *An. gambiae s.l.* in the study site with mortality rates below 35% for all sites tested for all 3 years of the study but, as discriminating dose assays can mask important changes in resistance strength (Toe et al., 2014) a more quantitative assay was needed to measure the strength of this resistance for the purposes of this chapter. This is

challenging as bioassay data are notoriously variable being influenced by biases in sampling, larval rearing conditions and temperature and/or relative humidity (reviewed in (Ranson and Lissenden, 2016)). In addition the time of year at which the bioassays are performed can have an important impact on the results, particularly in areas with intense use of insecticide in agriculture such as Banfora, where susceptible genotypes may be less common after the crop spraying season (Ranson et al., 2009). Another important variable is the species composition. A previous study in Burkina Faso found that the probability of surviving the diagnostic dose of permethrin varied markedly across four sites (Badolo et al., 2012). However, when the results were stratified by members of the *An. gambiae* complex, a remarkably similar survival rate was observed across all four sites for a single species *i.e.* the difference in bioassay mortality rates between sites was largely explained by variations in species composition (Badolo et al., 2012), with *An. gambiae s.s.* being more resistant than *An. coluzzii*. Although in the current study *An. gambiae s.s.* predominated in the majority of larval collections in 2013 a high proportion of the collection from Tiefora Centre were *An. coluzzii* and this may have contributed to the lower resistance prevalence at this sampling point. Low sample sizes for *An. coluzzii* in the bioassays precluded a statistical analysis of the impact of species distribution on bioassay results in this chapter so all results are presented for *An. gambiae s.l.*

Two alternative quantitative bioassays were used during the 3 years: WHO tube assays in which the variable was time to exposure and CDC bottle bioassays in which insecticide dose was variable. This was logistically very challenging given the large number of mosquitoes required for each of these methods, and resulted in critical gaps in data points and added another layer of complexity to the data analysis. However it is important to note, that in 2013 when this study was initiated, there was no consensus on how to measure the strength of resistance and one of the secondary objectives of this work was to compare results using different methodologies (Bagi et al., 2015). As reported for laboratory strains, the estimates of resistance ratios for the Banfora population varied greatly depending on the quantitative variable (exposure time or dose) with resistance ratios compared to a standard susceptible strain being

approximately 10-fold higher when the LC50 value was used as the estimate of resistance strength than when LT50 was used. Clearly further work is needed to agree on the most appropriate method of quantifying resistance.

The cone bioassay results provide an indication of the impact that this level of resistance could have on standard permethrin-treated LLINs. Extremely low levels of mortality were observed after a 3 min exposure to either conventional Olyset nets, Olyset Plus or Olyset Duo. Although concerns have been raised about the suitability of cone bioassays to measure performance of Olyset nets (Siegert et al., 2009), results using Permanet 2.0, which contains deltamethrin, were equally concerning. Measures of mortality almost reach the 80% limit recommended by the WHO when Permanet 3 was tested (deltamethrin with the addition of PBO), but in general results suggest that none of the tested nets can control satisfactorily malaria mosquitoes from the study site.

4.4.2 Impact of Olyset Duo on permethrin resistance levels

The RCT involved distribution of Duo in a step-wedge design, ensuring that the coverage gradually increased throughout the two years of the study. Ideally, the analysis of the time-response data would have been better if performed independently for mosquitoes collected from each cluster and time since introduction of Duo, but there were insufficient data points for this analysis. Xi^2 analyses of the standard WHO susceptibility assays in three health centres showed no difference in mortality through the three years of the trial. Because of the availability of data for identical time exposures before and after the introduction of Duo, a BLR aimed to explain the mortality caused by 60, 90 and 120 min exposure to permethrin WHO tests was done only for Tiefora Centre.

The distribution of Duo did not cause any impact on the susceptibility of Tiefora Centre mosquitoes to permethrin ($p=0.7$) evaluated with WHO susceptibility tests. Interestingly, exposing mosquitoes to permethrin longer (90 and 120 min) caused a significant decrease in survival; this effect would have been missed if only the standard 60 min exposures to permethrin would have been done. Recently it has been discussed that the diagnostic dose and exposure time currently recommended by WHO may not be the most appropriate given the unprecedented levels of insecticide resistance (Toe et al., 2014, Bagi et al.,

2015), therefore changes in this phenotypic trait may be underestimated due to low mortality. This missed information can be very important to understand the evolution of insecticide resistance in vector control programs or trials, so further information on different effects of exposure times should be investigated in the future. This analysis was based on three years of WHO susceptibility bioassays carried out during the RCT in a single village, therefore since no simultaneous comparable data were obtained in other villages or health centres these results must be interpreted cautiously. Further resistance monitoring in subsequent years would be needed to conclusively demonstrate any impact of Duo on permethrin resistance.

Use of two different bioassay methodologies, and logistical challenges which led to variations in the selection of villages used for larval collections between years, both limited the conclusions that can be drawn from this study. Further data from a control site, in which Duo was not introduced would also be needed to determine whether the introduction of a net containing PPF really can be an effective resistance management tool.

4.4.3 Molecular mechanisms of insecticide resistance

The use of molecular markers of permethrin resistance could help overcome some of the challenges in the use of bioassays to accurately quantify resistance (Donnelly et al., 2016). The most widely used pyrethroid resistance marker detects mutations in the 1014 codon of the pyrethroid target site, known as kdr alleles. The 1014F kdr allele was found at allelic frequency > 90% throughout the study site. There was no significant variation in the allelic frequency of either the 1014F or the 1575Y kdr allele from 2013 to 2015. Mosquitoes genotyped belonged to the negative controls unexposed to insecticides, so it was not possible to establish the contribution of these kdr mutations to the resistance phenotype in this study but previous studies have shown an association between the presence of both of these alleles and pyrethroid bioassays (Donnelly et al., 2009, Jones et al., 2012) but not bednet performance (Strode et al., 2014). Theoretically one of the effects of Duo on the mosquito populations would be the reduction of insecticide resistance alleles. However, in

populations where the wild type allele is already rare, and where compensatory mutations may have already occurred reducing any fitness cost of the resistance mutation, this may take place over a very long time period and be difficult to detect.

Microarray experiments were conducted to compare the transcriptome of mosquitoes from the field site with laboratory susceptible strains in order to identify any other candidate genes that might be associated with resistance. The results presented in this chapter constitute a preliminary analysis and quantitative PCR is ongoing to confirm the upregulation of these genes in the Banfora colonies at LSTM and to test for an association between their expression and resistance. Nevertheless it is interesting to note the presence of several candidate genes strongly associated with pyrethroid resistance in other populations amongst the candidate gene lists generated in this study.

The CYP6P3 and CYP6Z2 genes, which were over-expressed in all the microarrays plus in previous studies on resistant populations from Vallee du Kou and Tengrela, have been widely linked to insecticide resistance in *An. gambiae* populations across sub-Saharan Africa and clearly linked with pyrethroid resistance (Djouaka et al., 2008, Muller et al., 2008, Abdalla et al., 2014, Yahouedo et al., 2016). The CYP4G16 gene was also reported to be overexpressed by Toe *et al.* in resistant populations from Burkina Faso (Toe et al., 2015), and recently was associated with cuticular hydrocarbon (CHC) production in *An. gambiae* (Balabanidou et al., 2016). It is hypothesised that over expression of this P450 may reduce the rate of penetration of insecticides and the potential role of reduced penetration in the Banfora populations is supported by the finding that several cuticular proteins (CPs) were overexpressed in the multiple comparisons in this study. The CPLCG family has been previously found over-expressed in pyrethroid resistant populations (Vontas et al., 2007, Awolola et al., 2009), and additionally its potential role in penetration resistance has been linked with overexpression in mosquito legs (Vannini et al., 2014). Studies on the association between expression of this subfamily and resistance are ongoing.

The ABC-binding cassette transporter proteins (ABC) are widely studied in vertebrates due to their well characterised role in drug resistance, however they are less well studied in arthropods (Dermauw and Van Leeuwen, 2014). Recent studies have demonstrated a role for insect ABCs in the tolerance to the toxins of *Bacillus thuringiensis* (Tabashnik, 2015) and in permethrin detoxification in *An. stephensi* larvae (Epis et al., 2014). The ABCB4 gene that was found upregulated in pyrethroid resistant populations in this study was also identified in resistant *Anopheles gambiae* from Cameroon (Fossog Tene et al., 2013) and in resistant *Ae. aegypti* (Bariami et al., 2012), making it a potential good candidate for further characterisation.

The microarray data alone do not enable us to identify changes in gene expression associated with the Duo distribution. Further data points would provide more evidence of the effect of Duo on the dynamics on the expression of candidate genes. Also, the development of DNA markers for those candidate genes would provide a cheaper and more widely used tool to monitor the effect of interventions in the expression levels.

Chapter 5 Sterilising effect of Olyset Duo on *Anopheles gambiae s.l.* after field implementation in Banfora district, Burkina Faso.

5.1 *Introduction*

Olyset Duo (Duo) is a combination LLIN that contains the Juvenile Hormone mimic pyriproxyfen (PPF) and the pyrethroid insecticide permethrin. The rationale in the design of the Duo LLIN is that mosquitoes surviving exposure to the insecticide component of the LLIN will be sterilised by PPF, preventing the transfer of resistance genes to progeny. Although field and semi-field trials have evaluated the efficiency of PPF as a pupacide (using the formulation Sumilarv®0.5G, Sumitomo Chemicals Co., Ltd.), its efficiency as a mosquito chemosteriliser has been only addressed in laboratory and experimental huts, and very recently, a small scale field evaluation (Kawada et al., 2014, Ngufor et al., 2014, Djenontin et al., 2015, Koffi et al., 2015).

This study was part of a clinical trial studying the efficiency of Duo compared to standard Olyset nets in the Banfora district of Burkina Faso. A step wedge design was implemented so the initial total coverage by Olyset nets was replaced gradually by Duo in randomly assigned clusters of villages, until the end of the trial when Duo coverage was total (Tiono et al., 2015).

The objective of the study was to test the hypothesis that Duo LLINs can diminish the reproductive output of *Anopheles* mosquitoes under field conditions. To achieve this, the oviposition rates and ovary morphology of mosquitoes collected in houses with Duo or no bednet was firstly investigated in Tiefora Centre village. After this exploratory evaluation, the effect of Olyset nets replacement by Duo was monitored at key points over two years in five villages belonging to three different treatment clusters.

5.2 *Methods*

5.2.1 Study site and Randomised Controlled Trial

Six sentinel sites within three Health Centres in the Cascades region of Burkina Faso were selected, as shown in Table 5.1. Olyset nets were distributed to all households in May - June 2014 and then were replaced by Duo from June 2014 to September 2015 in a stepped wedge fashion (Tiono et al., 2015). Mosquito collections were performed before and after the replacement of Olyset by Duo nets in five villages except in Tiefora Centre, where collections took place in October 2014 when Duo were already distributed. In this case, instead of a before-after Duo strategy, mosquitoes were collected in houses with no LLIN deployment or with Duo. Sampling dates for each village related to the distribution of Duo are shown in Figure 5.1. Mosquitoes from Tengrela, a village that was not part of the RCT but that is approximately 5.6 km away from the study site, were collected as a negative control. The collectors checked the LLINs deployed in the houses to confirm that no Duo were present in any house in this village.

Table 5.1 Geographic information of the villages where mosquitoes were collected for the study. Geographic information of the villages where mosquitoes were collected for the study. Tengrela village was selected as a negative control, without intervention.

Health Centre	Village	Coordinates
Tiefora	Tiefora Centre	10˚37'54.02''N; 4˚33'22.85''W
	Djomale	10˚33'17.24''N; 4˚22'41.14''W
	Pont Maurice	10˚38'26.71''N; 4˚29'41.62''W
	Sikane	10˚34'27.49''N; 4˚22'38.16''W
Kankounadeni	Naniagara II	10˚32'9.15''N; 4˚40'7.84''W
Koflande	Bakaridjan II	10˚24'26.34''N; 4˚33'44.78''W
Banfora regional hospital	Tengrela	10˚38'7.53''N; 4˚48'48.35''W

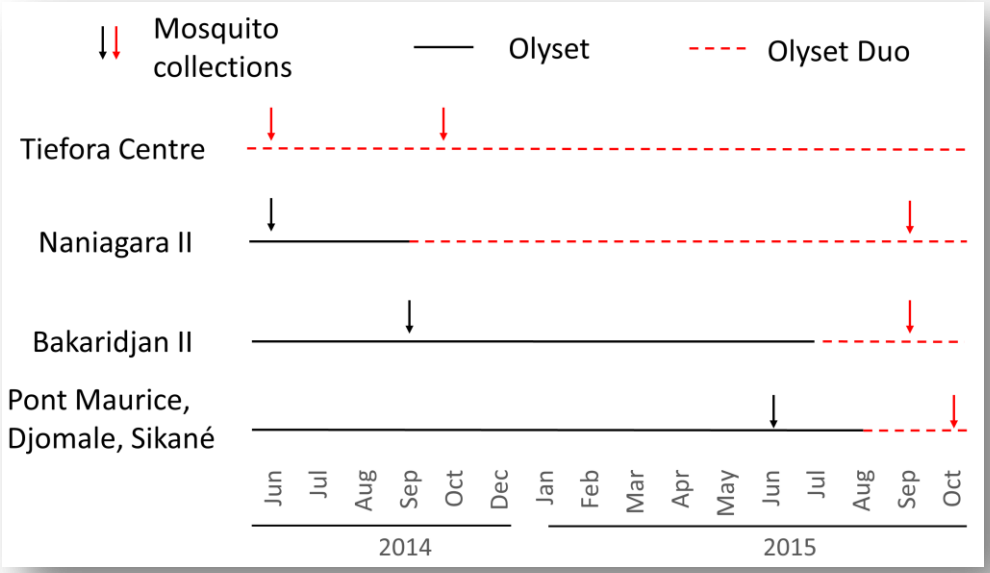


Figure 5.1 Dates of mosquito collections and distribution of Olyset/Olyset Duo nets in the six sentinel sites. The continuous black lines represent the distribution periods of Olyset nets, while the dashed red lines represent Olyset Duo. Arrows represent the months where collections were done before (black) and after (red) Olyset Duo distribution. Note that 'Tiefora Centre' in this scheme refers to the village, while 'Tiefora Health Centre', also referred to widely in this thesis but not in this scheme, is the wider jurisdiction that comprises villages such as Pont Maurice, Djomale, Sikane and Tiefora Centre itself. See Table 5.1 above for further details.

5.2.2 Mosquito collections

Female bloodfed anopheline mosquitoes were collected inside houses in the study villages. Collections were firstly done in the village of Tiefora Centre, where Duo was already distributed (Figure 5.1 above). Because the Duo use was low in this village, collections were done simultaneously in houses with Duo and houses with no bednets deployed, and those groups analysed separately.

Secondly, collections were done in the other sentinel villages in a before/after Duo fashion. For the 'baseline' collections (i.e. the collections done when only Olyset nets were distributed) mosquitoes were captured in every house where the collectors were allowed to enter and meta data (descriptive information

about the demography, architecture, presence of animals and other contextual observations that may help to explain the outcomes) were not collected; for the follow up 'intervention' collections (*i.e.* Duo distributed) only houses with evident deployment of Duo were entered. In this case information about the name of the chief of the village, the name of the chief of the house, number of people sleeping, the presence of animals around the houses, an individual picture of the house and the code of the nets deployed were recorded.

Collections were done early in the mornings, starting at 6 am. Inhabitants of the village were requested to keep the house windows closed until collections were finished. Mosquitoes were aspirated into plastic containers using Prokopacks (Vazquez-Prokopec et al., 2009) powered by 12 V batteries from all possible resting places: roofs, under beds, walls, clothes hanging in wires, decorations and furniture. Then they were transferred gently into mosquito cages and transported to the insectaries in Banfora Regional Hospital. After each collection, the plastic containers and cages were washed thoroughly to avoid contamination. Collections were repeated until at least approximately one hundred females had been assessed for oogenesis from each village in each period of collections.

5.2.3 Oviposition assays

Bloodfed, half-gravid and gravid mosquitoes were separated for the oviposition assays, and non-bloodfed, dead gravid and males were preserved in silica gel. The presence of other mosquito species was negligible so it was not recorded. In the oviposition assays, the selected mosquitoes were transferred to individual cell culture tubes with a wet piece of filter paper as an oviposition substrate. Mosquitoes that died during the subsequent period were removed from the experiment. A piece of cotton wool moisturized with 10% sucrose was available for the mosquitoes. Oviposition was recorded every day, and the mosquitoes that did not lay eggs by the sixth day were dissected to isolate the ovaries. The morphology of the ovaries was noted and, in case of healthy eggs, the number of eggs produced recorded (for the full methodology see Chapter 2 numeral 2.2.3.1). Ovaries were scored as abnormal when the eggs were bubble-like, presented irregular shapes and were non-detachable; normal ovaries were

scored when the eggs presented the characteristic oval shape and were easily detachable from the ovary envelope (Figure 2.6, Chapter 2). All mosquitoes collected from the houses were labelled individually and preserved in silica gel.

5.2.4 Egg hatch assays

Individual egg batches were transferred to plastic pots where the hatching and the development of larvae was monitored. Numbers of larvae reaching the second instar were recorded as an estimate of hatchability. The detailed methodology was described in the numeral 2.2.3.2 in Chapter 2.

5.2.5 Species identification

A minimum of 28 and a maximum of 72 *An. gambiae s.l.* mosquitoes per village were identified to species level by PCR. For collections done before the intervention, mosquitoes with normal ovaries or oviposition were selected; after the intervention, the mosquitoes selected for species identification were selected from both those with normal ovaries / oviposition and mosquitoes with abnormal ovaries in approximately equal numbers. DNA was extracted by heating two mosquito legs at 95°C in 100 µl of 1X PCR buffer diluted in the laboratory for 30 min and species identification PCR performed according to Santolamazza (Santolamazza et al., 2008) (description of the methodology in the numeral 4.2.3.2; electrophoresis figure 4.2; PCR conditions Table A3 in the appendix).

5.2.6 Blood source identification

Bloodfed mosquitoes that died as a result of the collection method or the transportation to the insectaries were preserved in silica gel and transported to LSTM laboratories in the UK for BM analysis. Mosquitoes collected in Pont Maurice, Sikane and Djomale before and after the distribution of Duo were tested. Abdomens were separated from the rest of the carcass and the DNA was extracted by the LIVAK method described previously (Chapter 4, numeral 4.2.3.1). A PCR amplifying a fragment of the Cytochrome b followed and the blood source was characterised by electrophoresis according to Kent and Norris (Figure 5.2) (Kent and Norris, 2005).

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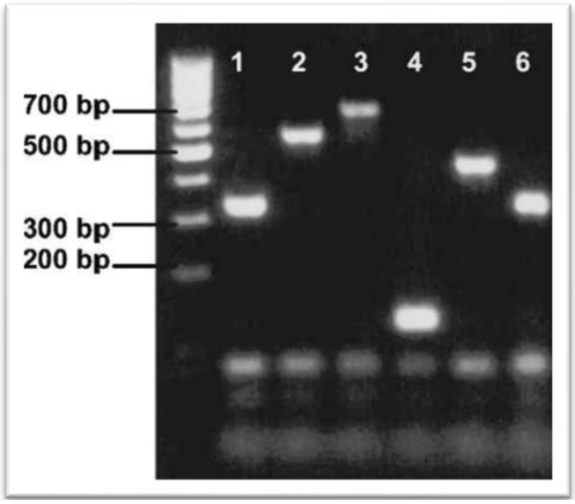


Figure 5.2 Guide for the identification of blood source in mosquitoes

Ethidium bromide agarose gel showing the expected size of the cytochrome b fragments amplified by PCR. Lane 1 corresponds to human (334 bp), 2 to cow (561 bp), 3 to dog (680 bp), 4 to goat (132 bp) and 5 to pig (453 bp). The DNA ladder is a 100 bp ladder. Figure obtained and modified from Kent and Norris, 2005 (Kent and Norris, 2005)

5.2.7 Statistical analyses

5.2.7.1 Descriptive analyses of proportions

Z-tests were used to compare the proportions of dead, egg laying and dissected mosquitoes after collections. All the Z-tests were done in the VassarStats computational website (http://vassarstats.net/). The SPSS software (IBM) was used for the rest of statistical analyses.

5.2.7.2 Odds of collecting non-bloodfed mosquitoes before and after Olyset Duo

Only mosquitoes collected within Tiefora Health Centre in 2015 (Pont Maurice, Djomale and Sikane) were used in this analysis because of physiological status data availability. A Binary Logistic Regression (BLR) addressing the effect of 1) location (villages), 2) the presence of Olyset Duo and 3) the interaction between those two variables on the odds that mosquitoes collected were non-bloodfed

was done. One random order for entering the villages into the model was selected, and then kept consistent throughout the analyses. This allocation was randomly assigned. The 'bloodfed' category included bloodfed, semi-gravid and gravid mosquitoes.

5.2.7.3 Egg retention odds

A BLR was used to assess the egg retention odds in mosquitoes collected in the presence of Duo. Separate analyses were conducted for Tiefora Centre, where all data collected was under Duo coverage, and the rest of villages where the arms of the study were before-after Duo. The explanatory variables were 1) location (not for Tiefora Centre) and 2) the absence/presence of Duo; the binary outcome was either oviposition or egg retention. Dissected mosquitoes that developed eggs, normal and abnormal, were considered under the 'egg retention' category. The 'location' variable not only accounted by the geographical distance, but it also marked the time between collections. In Naniagara the time between collections was of 15 months, in Bakaridjan of 12 months and in Pont Maurice, Djomale and Sikane of 3 months. The second round of collections were performed 12 (Naniagara village only) or 2 months after Duo distribution (see Figure 5.1 above).

5.2.7.4 Olyset Duo effect on normal oogenesis odds in Tiefora Centre

This analysis aimed to answer the following question: are mosquitoes collected in houses with Duo more likely to develope abnormal ovaries than mosquitoes collected in houses without LLINs? This question was only addressed in Tiefora Centre collections (2014), because abnormal ovaries were found in both arms of the experiment ('No bednet' and 'Olyset Duo'); in the collections under the before/after Duo experimental design this could not be done because there were not abnormal ovaries in collections before Olyset Duo (total count = 0).

A BLR with location and the presence/absence of Duo as explanatory variables were used again, and the binary outcome was grouped as it follows: 1) normal oogenesis, which included mosquitoes that laid eggs and those which presented normal eggs after dissection, and 2) dissected mosquitoes that presented abnormal ovaries.

5.2.7.5 Olyset Duo effect on egg production

First, direct comparisons between the average oviposition sizes laid in the two arms of the trial (with or without Duo) within each village were done. The Kolmogorov-Smirnov test was used to test the normality of the oviposition batches and then the equality of variances was assessed by the Levene's test. Ovipositions were compared by Independent Samples T-tests, and Standard Error of the Mean (SEM) were provided.

Second, a multiple linear regression (MLR) addressing the effect of 1) location, 2) egg retention status and 3) the presence of Duo was done. The outcome was the count of individual egg batches laid or developed normally (i.e. eggs laid and eggs counted after ovary dissections). Mosquitoes that showed abnormal ovaries were not used in this analysis.

5.2.7.6 Egg hatch rates

The hatchability of eggs laid under the two arms of the study (presence or absence of Duo) were compared using Z tests.

5.2.7.7 Effect of mosquito species on the response to Olyset Duo

To investigate whether either *An. gambiae s.s.* or *An. coluzzii* species could affect the response of the mosquitoes to the sterilising effects of PPF, a BLR was done. The explanatory variables were location (villages) and species (*An. gambiae s.s.* and *An. coluzzii*). The data was taken only from the species identification from the 'intervention' collections, i.e. after Duo distribution; the reason is that in the baseline collections without Duo no mosquitoes presented abnormal ovaries, so the frequency of this would be null, not comparable. The outcome variable was normal oogenesis (pooling laid eggs and normally developed eggs) or abnormal oogenesis.

5.3 *Results*

5.3.1 House architecture

House architecture was variable in the different villages. The most common houses were built from mud bricks (Figure 5.3 A) with two rooms: one bedroom, usually with space only for the bed, clothes and personal belongings, and one 'living room' where children or visitors occasionally slept on the floor, and where a range of objects (*e.g.* bikes, pesticides, grains, batteries, clothes, containers, etc.) were stored. Where people slept in the living room, a net was normally deployed there too. Usually the bedroom had a single window, and the roofs were either made from corrugated iron or thatch (Figure 5.3). The other common house structure, particularly prevalent in Pont Maurice, was a single-room house with thatch roof (Figure 3B). Bigger houses made of mud or stone bricks were less common (Figure 3 C, D). Most of the houses (except the single-room) had a small additional room for bathing.

Figure 5.3 Typical house architecture in the study site. The most common types of houses in the study site were made of A) mud bricks and corrugated iron roof, two rooms B) mud bricks with thatch roof, single-room. Less common houses: C) Multi-room house with thatch roof; D) Multi-room house made of stone bricks and corrugated iron roof.

5.3.2 Demographics and presence of animals

The number of adults per housing structure ranged from 1.3 to 1.8 adults in different villages, and the number of children ranged from 1.1 to 2.4 (Table 5.2). Note that in this region polygamy is common, in which case the older wife will live with her children in a separate house. Also, elderly people and recently independent young adults usually live alone. Hence the numbers presented in Table 5.2 do not represent the size of the household, rather the number of people in the housing structure from which mosquito collections were made. Percentages of adults/children in the different collection sites varied were between 41% and 59%, picturing a relative uniformity between children and adults in the villages.

Table 5.2 Demographic data on the visited villages in 2015. Numbers of children and adults that slept in the house the night preceding the indoor collections in villages visited in 2015. Average of adults/children per house and percentages were based on the surveys done on the day of mosquito collections. Some houses were visited more than once, but for each time the reported number of inhabitants was entered.

Village	Adults	Children	Inhabitants	Houses*	Adults/house	Children/ house	% Adults	% Children
Bakaridjan	72	53	125	41	1.8	1.3	57.6	42.4
Naniagara	39	56	95	45	1.6	2.2	41.1	58.9
Pont Maurice	208	155	363	140	1.5	1.1	57.3	42.7
Djomale	49	57	106	37	1.3	1.5	46.2	53.8
Sikane	42	55	97	23	1.8	2.4	43.3	56.7

*Some of these houses were visited more than once.

The presence of animals was variable. Goats, chickens and dogs were abundant, although in some areas of Pont Maurice cows were the most common animal recorded. Notably, part of this village is inhabited by members of the Fulani tribe, historically a nomadic group, whose main economic activity is cattle farming. Sheep were also common, although more so in central villages such as Tiefora Centre. By the end of the rainy season predators usually get closer to the human settlements by camouflaging in the high weeds and preying

on chicken. According to the local guide, ophidic accidents were common in Bakaridjan with a rate of one snake or scorpion bite per week.

5.3.3 Mosquito collections

All indoor collections were done during the 2014 and 2015 rainy seasons. The first collections in 2014 in Tiefora Centre aimed to standardise the methods of collection and oviposition assays. For logistical reasons it was not possible to collect mosquitoes before the distribution of the Duo in this village and no meta data was recorded from houses for collections performed in. Instead mosquitoes were collected from any house where entrance was allowed.

Mosquitoes were found resting on the walls, roof, under furniture, on hanging clothes and in shady, cool places. The numbers of mosquitoes captured daily were highly variable ranging from no mosquitoes (common) to >100 bloodfed mosquitoes (uncommon). Most of the mosquitoes collected indoors were bloodfed or half-gravid female *Anopheles sp.*, although non-bloodfed females were also collected (Table A10, appendix). The number of houses visited and total number of mosquitoes collected in every village are shown in the Table 5.3. These data are not a measure of mosquito density; many houses were inaccessible at the times of sampling, some houses were visited on repeated occasions and the collectors were not the same during the whole collection period. The aim of these collections was to ensure an approximate number of 100 mosquitoes / village / period of collection that reached alive one of two endpoints: oviposition or dissection 6 days after collection. In total 2299 mosquitoes were collected, from which approximately 811 (35%) died immediately after the collections or during the experimental process (Table 5.3). A total number of 500 mosquitoes (21.7%) laid eggs, while 988 (43%) were dissected.

Table 5.3 Indoor mosquito collections and mosquito sample size. Table showing the dates and yields of indoor mosquito collections in six study villages. LLIN replacement refers to the date when Olyset nets were replaced by Olyset Duo nets. The number of houses visited is the total number of independent structures where collections were made; houses visited more than once were counted multiple times. The number of mosquitoes includes only female *Anopheles sp*. The detailed information about the mortality in the oviposition assay and the ovary dissections scoring is provided in the appendix (Table A11, appendix).

Village	LLIN replacement	Collection Date	Number of houses visited	Mosquitoes collected	Dead	Mosquitoes laying eggs	Dissected
Tiefora Centre	June 2014	October 2014*	12	95	33[+]	41	21
		October 2014**	14	90	13[+]	20	57
Naniagara	September 2014	June 2014	No info	92	4[+]	51	37
		Sept - Oct 2015	45	291	90	7	194
Bakaridjan	July 2015	September 2014	No info	202	18[+]	46	138
		Sept - Oct 2015	60	184	31	12	141
Pont Maurice	August 2015	June 2015	No info	213	104	88	21
		Sept - Oct 2015	140	220	104	15	101
Djomale	August 2015	June 2015	No info	361	242	99	20
		October 2015	37	117	12	5	100
Sikane	August 2015	June 2015	No info	239	104	101	34
		October 2015	23	195	56	15	124
Total			-	2299	811	500	988

*No bednet

**Olyset Duo

[+] Numbers not including the dead mosquitoes the day of collection

5.3.4 Olyset Duo effect in mosquito reproductive output: Tiefora Centre

5.3.4.1 Mosquito mortality, oviposition and dissection proportions

Mosquito collections in Tiefora Centre were done when Duo nets were already distributed, therefore sampling was done in houses with Duo nets or without any LLIN. Due to that basic difference with the rest of sites, these results are shown in a separate section. These sets of collections helped as a preliminary exploration of the possible sterilisation effects on mosquitoes, to standardise the experimental protocols and improve data collection. For instance, at this time no information was recorded about the physiological status of the mosquitoes collected (gravid, semi-gravid, engorged), the dead on the day of collection and the number of males. This was improved for the 2015 collections, when that information was recorded.

In the weeks immediately after distribution, usage of Olyset Duo in Tiefora Centre was lower than would have been expected under trial conditions (54%). This enabled mosquitoes to be collected simultaneously in houses with Duo or without any LLIN deployed (No bednet). In total, 86 and 58 bloodfed Anopheles were isolated in oviposition tubes after collection in houses with no net and in houses with Duo respectively. Mortality after mosquito isolation was 38.4 % in the ‘No bednet’ arm and 22.4 % in the Olyset Duo arm. Oviposition in the ‘No bednet’ arm was of 75%, significantly higher than the 45% oviposition in the Duo arm ($Z= 3.35$ $p< 0.001$); additionally, the percentage of mosquitoes with normal ovaries was significantly higher in the absence of Duo nets ($Z= -2.6$ $p< 0.01$) (Figure 5.4).

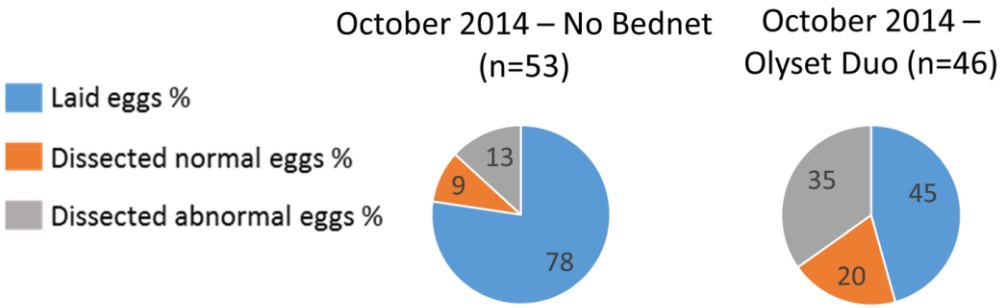


Figure 5.4 Oviposition rates and ovariy status of mosquitoes collected in Tiefora Centre in October 2014 (following Olyset Duo distribution). Pie charts showing the percentages of mosquitoes that laid eggs in the oviposition assay (blue), mosquitoes with normal and abnormal ovaries after dissection (orange and grey). Note that collections were done only in houses without any bednets or in houses deploying Olyset Duo.

5.3.4.2 Egg retention odds and oogenesis

The percentage of mosquitoes retaining their eggs (both normal and abnormal) five days after collection was 55 % in the Duo collections, higher than in the 'No bednet' collections where the retaining percentage was 22% (Figure 5.4, above). Mosquitoes collected in houses with Duo were 4 times (95 % CI 1.7 – 9.7; $p< 0.005$) more likely to retain eggs than mosquitoes collected in houses without bednets.

The rates of normal egg development were measured grouping the number of mosquitoes that laid eggs and the mosquitoes showing normal eggs within the ovaries after dissection. In the 'No bednet' arm 87 % of the mosquitoes developed normal ovaries, while this percentage was 65 % in the 'Duo' arm. Mosquitoes collected in houses with Duo were 3.5 times more likely to develope abnormal eggs (95 % CI 1.29 – 9.53; $p< 0.05$).

5.3.4.3 Oviposition size

The mosquitoes collected in houses without bednets laid an average of 115.7 eggs (n= 41; SEM= 8.8) whilst those collected in Duo houses laid 100.7 eggs (n= 20; SEM= 12.1) with no significant difference between these means (p= 0.33). This data was not modelled because it did not satisfy the assumption of correlation between the explanatory variables (retention status, intervention)

and the outcome (egg batch size) (Pearson's correlation coefficient: -0.031 and -0.197 respectively for the predictors; cut point: +/-0.3).

5.3.4.4 Egg hatchability

Second instar larvae counts were considered as the estimate for egg hatch. 12% of the total eggs laid by mosquitoes from the 'No bednet' arm (n= 4453 eggs) hatched, while 4.4% of eggs hatched in the Duo arm (n= 2015 eggs). This difference was statistically significant (Z= 10, p< 0.001).

5.3.5 Olyset Duo impact on mosquito reproductive output: before and after Olyset Duo

This section describes the results obtained in the collections following the 'before and after intervention' two-arms design in five villages belonging to three different health centres as described in the methods (numeral 5.2.1; Table 5.1). All surviving mosquitoes two days after the collections were isolated in oviposition tubes. Oviposition was recorded for 4 days more (i.e. 6 days after collection), and then all surviving mosquitoes were dissected and ovary morphology scored.

5.3.5.1 Mosquito mortality and physiological status before and after the distribution of Olyset Duo

The total number of mosquitoes collected was 1107 before and 1007 after Duo. The mortality, including dead on the day of collection or during the oviposition assay, was higher before Duo than after Duo (Z= 6.47; p< 0.001) (see Figure 5.4 above). The majority of mosquitoes collected were bloodfed (Table 5.4), and small percentages were non-bloodfed and gravid. A Binary Logistic Regression (BLR) addressing the effect of the sites of collection (villages) and the presence of Duo on the mosquito physiological status showed that there was 2.9 times more chance of catching non-bloodfed mosquitoes after Duo than before (see the Odd Ratios in Table 5.5). The analysis also showed an effect of location, indicating that it was more likely to find bloodfed mosquitoes in Djomale and Sikane than in Pont Maurice (the reference village); furthermore, the interaction

between the presence of Duo and the location was only significant for Djomale, not Sikane (Table 5.5).

Table 5.4 Physiological status of the female Anopheles. Total percentages of bloodfed, gravid and non-bloodfed mosquitoes collected before and after the distribution of Olyset Duo. The 'bloodfed' entry includes the mosquitoes that had that status but died upon collection and the mosquitoes that were used in the oviposition assays.

Village	Intervention	n	Bloodfed	Gravid*	Non-bloodfed*
Naniagara	Before Duo	92	ND	ND	ND
	After Duo	291	83.2 %	7.6 %	9.3 %
Bakaridjan	Before Duo	202	ND	ND	ND
	After Duo	184	84.8 %	11.4 %	3.8 %
Pont Maurice	Before Duo	213	99.1 %	0	0.9 %
	After Duo	220	74.5 %	5.9 %	19.5 %
Djomale	Before Duo	361	94.5 %	0	5.5 %
	After Duo	117	80.3 %	5.1 %	14.5 %
Sikane	Before Duo	239	98.7 %	0	1.3 %
	After Duo	195	94.4 %	1.5 %	4.1 %

*Dead on the day of collection

Table 5.5 Binary logistic regression analysing bloodfed abundance. The model predicts the effect of location and the presence of Olyset Duo in the likelihood of capturing non-fed mosquitoes. In this model the first variable entered within each category ('Pont Maurice' and 'absence of Olyset Duo') was the reference for the following variable.

Explanatory variables	B	S.E.	df	Significance	Odd ratios
Location			2	0.005	
Djomale	-1.823	0.747	1	0.015	0.162
Sikane	-1.529	0.625	1	0.014	0.217
Intervention					
After Duo	1.064	0.349	1	0.002	2.898
Interactions					
Intervention * Location			2	0.025	
Effect after Duo by Djomale	2.18	0.81	1	0.007	8.842
Effect after Duo by Sikane	0.149	0.768	1	0.846	1.161
Constant	-2.836	0.23	1	<0.001	0.059

5.3.5.2 *Oviposition and dissections before and after the distribution of Olyset Duo*

In this section the description of the pooled data is presented first, then the differences between and within villages are explored. The percentage of mosquitoes that laid eggs before Duo (n= 385) was higher than after Duo (n= 54) (Z= 16.6; p< 0.001) (Figure 5.5). From the combined mosquitoes from both arms, approximately 5 % did not show any signs of oogenesis, and a further 5% did contain eggs but the morphology of the ovaries could not be clearly discerned (i.e. eggs were to small so they could be still in development, or simply no clear decision could be made upon dissections) (Figure 5.5). These mosquitoes were excluded from the subsequent analyses and the remaining mosquitoes were classified within three categories: 1) mosquitoes that laid eggs, 2) mosquitoes that retained eggs scored as normal after dissections, and 3) mosquitoes that retained eggs scored as abnormal after dissection.

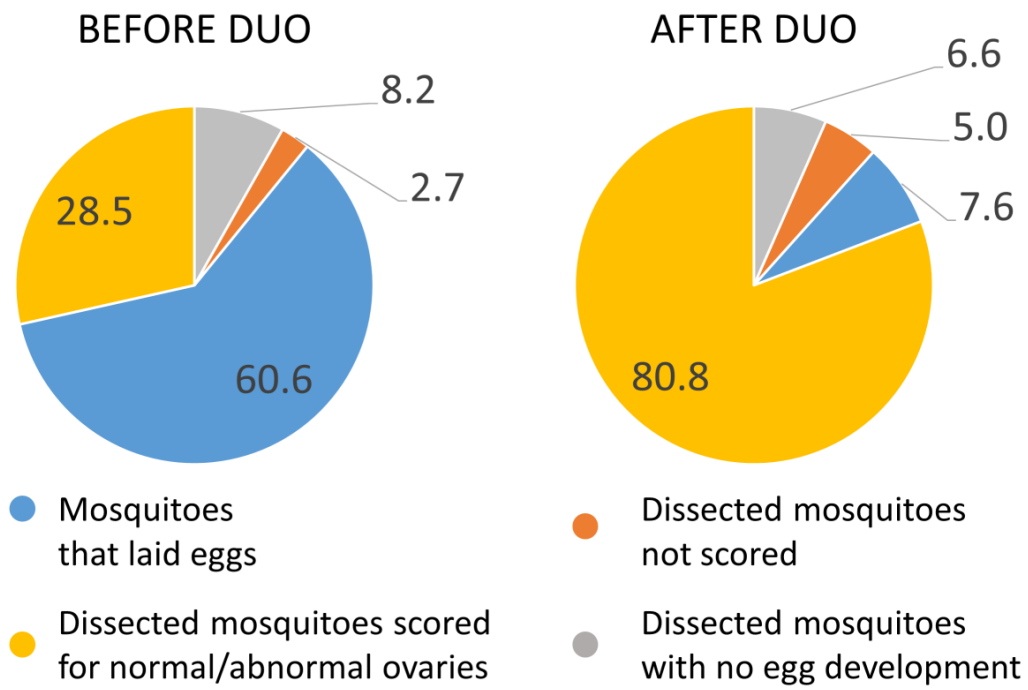


Figure 5.5 Percentages of mosquito survival, oviposition and oogenesis. Pie charts showing the percentages of mosquitoes that laid eggs, did not show oogenesis, were classified as unidentified or normal/abnormal before (n= 1107) and after (n= 1007) the distribution of Olyset Duo. The 'Dissected mosquitoes not scored' category cover dissected mosquitoes whose ovaries were not clearly identified as normal or abnormal.

Of those mosquitoes which fit within the categories mentioned above, 75% laid eggs while 25% retained them and presented normal oogenesis in the collections before Duo (n=515). No mosquitoes showed abnormal ovary development at this time. In the oviposition assays after Duo distribution, 8.6% mosquitoes laid eggs, 43.7% retained eggs with normal oogenesis and 47.7% showed abnormal oogenesis (n= 631). Mosquitoes from Tengrela village, a site close to the study site but not involved in the Duo trial, were collected simultaneously to the villages with Duo (October 2015). All the mosquitoes from this site showed normal oogenesis. Approximately half of the mosquitoes laid eggs (51.6%) while the rest were dissected (48.4%)(n= 31).

The proportion of mosquitoes collected from different villages that laid eggs ranged from 53% to 85% before the intervention and from 3.8% to 14% after the intervention (Figure 5.6). All ovaries dissected from mosquitoes prior to the Olyset Duo distribution (n dissected= 130) had normal morphology whereas after the intervention 43 - 62 % were scored as abnormal (n dissected= 577). Before-after Duo Z-tests within each village showed that the average of mosquitoes laying eggs was statistically different ($p< 0.001$).

5.3.5.2 *Egg retention odds before and after the distribution of Olyset Duo*

Mosquitoes collected in houses with Duo presented 61 times more likelihood of retaining eggs than mosquitoes collected previously in houses with Olyset nets ($p< 0.001$) (Table 5.6). The location of collections also influenced significantly the egg retention odds: in Bakaridjan the odds of egg retention (Odds Ratio: 4.7; $p< 0.001$) were significantly higher than in Naniagara, while in Djomale the odds were lower (Table 5.6). In Pont Maurice and Sikane, where Duo nets were deployed approximately 1 month before collections, the odds for egg retention were negative compared to Naniagara, the reference village (with Duo deployed for more than 1 year). Interestingly, Bakaridjan showed a strong significant interaction as a location with the presence of Duo ($p< 0.001$, OR= 0.08) (Table 5.6).

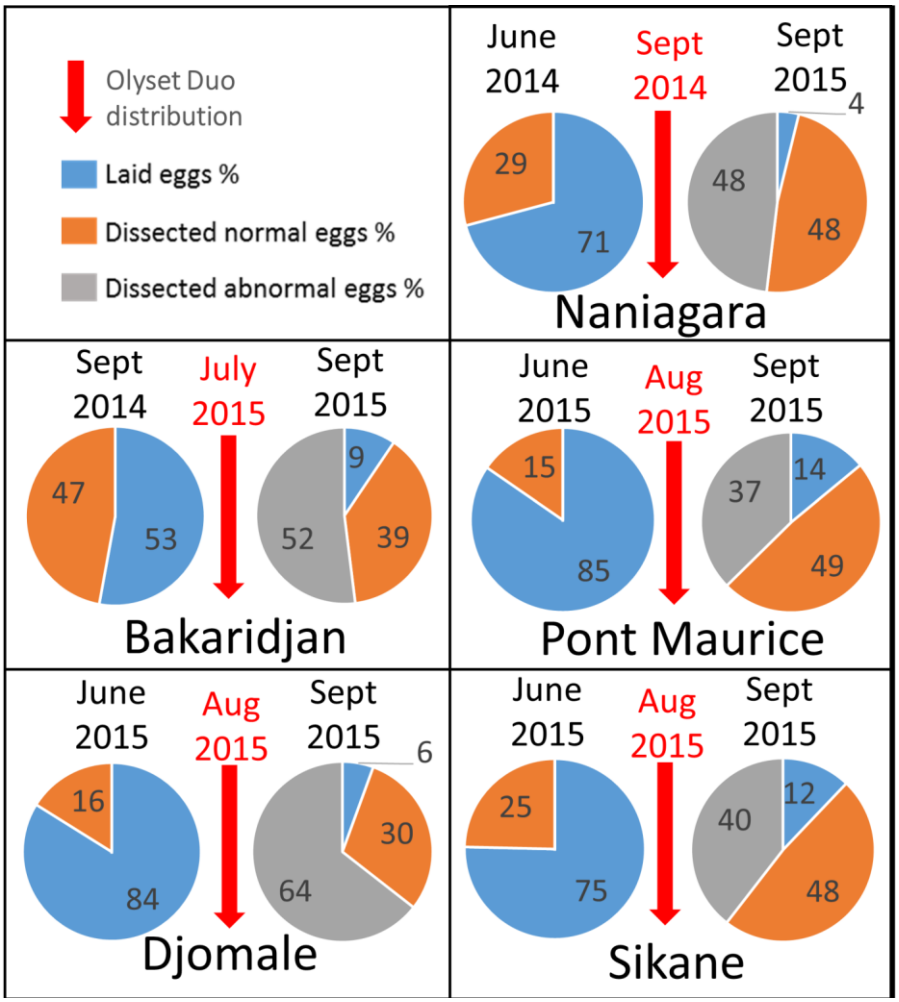


Figure 5.6 Oviposition rates and ovarian status of mosquitoes collected in villages before and after the distribution of Olyset Duo. Pie charts show the percentages of mosquitoes that laid eggs (blue), the mosquitoes that showed healthy ovaries after dissections (orange) and the mosquitoes that showed effect of pyriproxyfen (grey). In Naniagara (n=72 before Duo; n=183 after Duo) the time after the distribution of Duo was approximately 12 months, in Bakaridjan (n=87 before Duo; n=127 after Duo) of 3 months and in Pont Maurice (n=104 before Duo, n=107 after Duo), Djomale (n=118 before Duo, n=90 after Duo) and Sikane (n=134 before Duo, n=124 after Duo) of 1 month. The dates of collection for each dataset are shown over each pie chart. Dates of Olyset Duo distribution are shown between paired pie charts in red (red arrow).

Table 5.6 Effect of Olyset Duo and location of collections on egg retention. Results of a Binary Logistic Regression modelling the contribution of the intervention (presence of Olyset Duo) and the location of collections (villages). Naniagara Odd ratio is considered '1' as a reference for comparing with the 95 % CI of the other villages.

Explanatory variable	B	S.E.	df	Significance	Odd ratios
Vilage			4	<0.001	
Bakaridjan	1.558	0.316	1	<0.001	4.752
Pont maurice	-0.646	0.362	1	0.075	0.524
Djomale	-0.817	0.365	1	0.025	0.442
Sikane	-0.231	0.328	1	0.48	0.793
Intervention					
Intervention(1)	4.112	0.465	1	<0.001	61.061
Interactions					
Intervention * Village			4	<0.001	
After Duo by Bakaridjan	-2.532	0.584	1	<0.001	0.08
After Duo by Pont Maurice	-0.765	0.598	1	0.2	0.465
After Duo by Djomale	0.426	0.702	1	0.544	1.531
After Duo by Sikane	-1.001	0.576	1	0.082	0.368
Constant	-0.887	0.259	1	0.001	0.412

5.3.5.3 *Oviposition size and egg hatchability*

The average size of the egg batches ranged from 105 to 146 eggs/ mosquitoes before Duo, and between 61 and 102 eggs/mosquito after Duo. Average oviposition in all villages except Bakaridjan ($p= 0.89$) showed a significant reduction ($p<0.001$ for Naniagara and Sikane, $p< 0.05$ for Pont Maurice and Djomale) when mosquitoes were collected in houses with Duo compared with Olyset nets (i.e. after the distribution of Duo) (Figure 5.7). Mosquitoes collected in Tengrela village, the negative control site, laid 75.9 eggs/mosquito (n= 16).

To analyse the overall effect of Duo on oviposition size, data was pooled according in Olyset or Duo collections (before and after Duo). The mean number of eggs was 133.7 (n= 385; SEM= 3) with Olyset and 85.1 (n= 54; SEM= 8.1) eggs with Duo, and this difference was significantly different ($p< 0.001$).

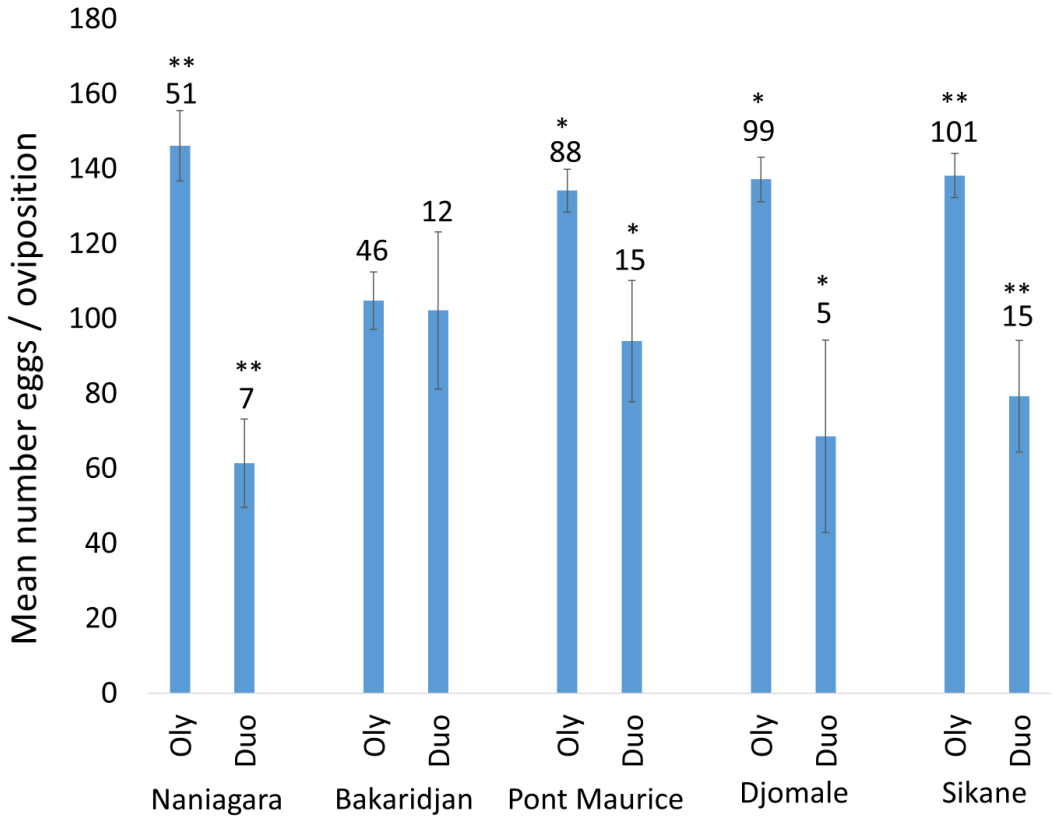


Figure 5.7 Mean number of eggs before and after the distribution of Olyset Duo. Histogram showing the mean number of eggs laid by mosquitoes collected in different villages where Olyset (Oly) and Olyset Duo (Duo) were deployed (mosquitoes laying no eggs were removed from this analysis). Numbers over each bar indicates sample size and the error bars shows the Standard Error of Mean (SEM). Significant differences within each village are shown over the sample size numbers as it follows: $p< 0.05$*, and $p<0.001$**.

A total number of 43195 eggs from mosquitoes collected in the baseline, and 4598 eggs collected from mosquitoes in Duo houses were used in the hatchability assays, and the overall hatch rate was 33.8% and 29.5% respectively. This difference was significant statistically ($p< 0.001$). Hatch rates were highly variable between sites, ranging from 28.9% to 72% in the collections before Duo and between 10% and 39% in the collections after Duo (Figure 5.8). Hatch rates were significantly lower in egg batches obtained from mosquitoes collected in houses with Duo in all villages except in Sikane (Figure 5.8). The significance of this difference was higher in Naniagara, Bakaridjan and Pont Maurice ($p< 0.001$) than in Djomale ($p< 0.05$).

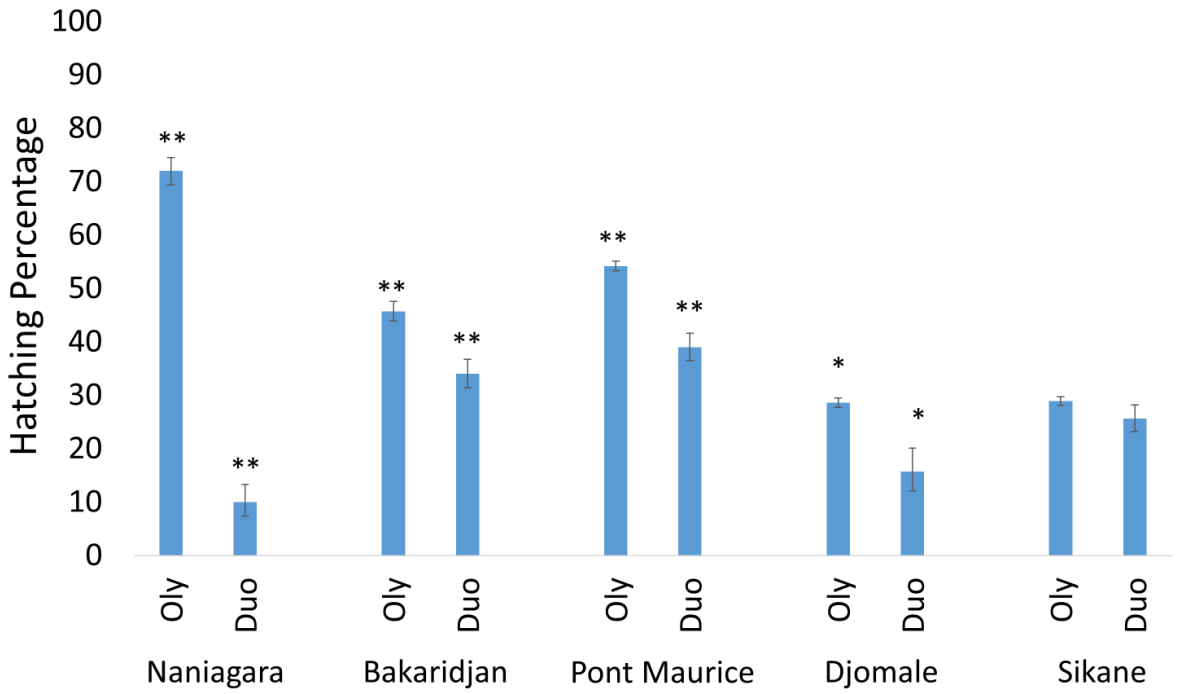


Figure 5.8 Hatch rate in the oviposition assays. The hatch rate was measured as the percentage of eggs that reached second instar over the totality of eggs laid. In the horizontal axis 'Oly' represents collections done when Olyset nets were distributed and 'Duo' refers to collections done after Olyset Duo nets were distributed. Error bars: 95% CI. Significant differences within each village are shown over each bar as it follows: *= p< 0.05, **= p<0.001.

5.3.5.4 *Species abundance in the An. gambiae s.l. complex*

An. gambiae s.s. was predominant in all indoor collections except in 2014 in Tiefora Centre, where it accounted for 21.4% of the mosquito samples in October 2014 (Figure 5.9). *An. arabiensis* was found indoors in three of the six sites but in very low numbers, the highest proportion in Sikane (June-July 2015) (Figure 5.9).

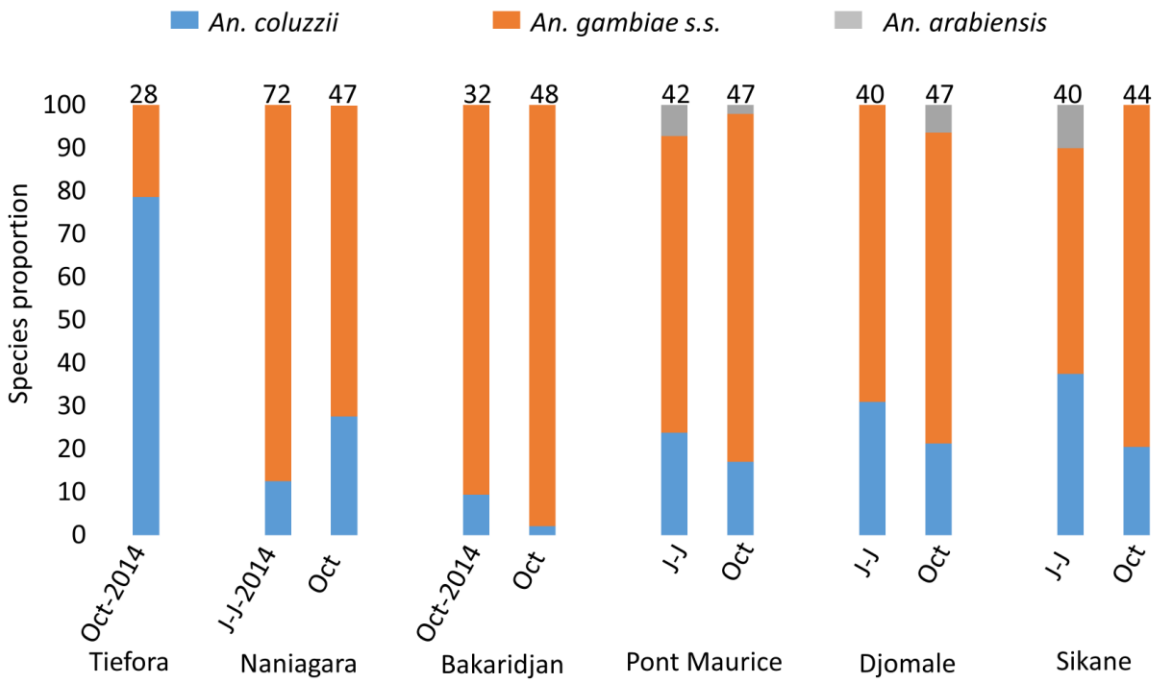


Figure 5.9 Species composition of mosquitoes collected indoors. Cumulative histogram showing the species composition of the sentinel sites collected without (first bar/village) and with Olyset Duo (second bar/village). The numbers over the bars are the sample size of mosquitoes screened. J-J: June-July; Oct: October. All collections were done in 2015 except the ones marked as 2014.

A BLR was done to assess the contribution of location and species (*An. coluzzii* and *An. gambiae s.s.*) on the odds of developing abnormal ovaries when mosquitoes were collected in houses with Duo. The model showed that the odds that both species develop abnormal ovaries when collected in Duo houses are not statistically different (p= 0.27).

5.3.5.5 *Bloodmeal source*

Bloodfed mosquitoes were particularly vulnerable to damage from the Prokopack aspirators. Any dead mosquitoes that were visibly bloodfed were stored immediately in silica gel for blood source identification. In total, 144 bloodfed mosquitoes collected before and after the distribution of Duo were tested for blood source (72 each time). The PCR had a low success rate (47.9%) but of the bloodmeals successfully completed, 81% had fed on humans followed by 16% on cows (Table 5.8). No bloodmeals on goats, sheep or pigs

were detected. The highest proportion of mosquitoes feeding on cows was in Pont Maurice, the study village with the highest numbers of cattle.

Table 5.7 Blood meal sources of mosquitoes collected indoors in 2015. Mosquitoes that died immediately after collections in Sikane, Pont Maurice and Djomale were preserved in silica gel and the blood source characterised.

Village	Olyset Duo distribution	n	Human	Cow	Human/Dog
Pont Maurice	Before	14	10 (71.4%)	4 (28.6%)	0
	After	7	4 (57.1%)	3 (49.2%)	0
Sikane	Before	12	10 (83.3%)	2 (16.7%)	0
	After	18	15 (83.3%)	1 (5.6%)	2 (11.1%)
Djomale	Before	6	5 (83.3%)	2 (16.7%)	0
	After	12	12 (100%)	0	0
All villages	Before	32	25 (78.1)	7 (21.9)	0
	After	37	31 (83.2)	4 (10.8)	2 (5.4)

5.4 *Discussion*

The AvecNet Olyset Duo clinical trial aimed to determine the efficiency of this new tool in reducing clinical malaria. Although, at the time of writing the final outcomes from this trial are still pending, this chapter reports on the impact of Duo nets on mosquitoes in the field site.

In this study we provide evidence that Duo LLINs can exert a significant negative effect upon different factors of the reproductive output of wild populations of anopheline mosquitoes. This conclusion is based on a number of key findings such as the high rates of mosquitoes with impaired oogenesis, reduced oviposition size and reduced egg hatch rates found in mosquitoes collected under Duo in comparison with Olyset.

5.4.1 Mosquitoes collected in houses with Olyset Duo show a drastic increase in egg retention

The odds ratios of egg retention in mosquitoes collected in Duo houses showed a dramatic 61 times increase. Considering specific village results, at least 91% (Bakaridjan) of mosquitoes collected in houses deploying Duo did not lay eggs compared to 25% (Sikane) in the same villages prior to Duo distribution. Direct examination of ovaries found that 48% of mosquitoes collected in houses with Duo were sterilised (defined here as mosquitoes showing morphological abnormalities in oogenesis) whereas no sterile mosquitoes were observed following dissection of mosquitoes in the first collection round. Importantly, this sterilisation effect was also significant for mosquitoes collected in houses that had Duo nets for over a year (Naniagara village). To eliminate changes unrelated to the presence of Duo, mosquitoes from Tengrela, a neighbouring village not involved in the trial, were dissected in 2015 following the same procedure as in the Duo villages; all mosquitoes from Tengrela presented normal oogenesis.

Oviposition behaviour has been linked to a combination of tactile, olfactory and visual cues (Bentley and Day, 1989, Dhileepan, 1997). Attractive and repulsive volatiles for gravid *An. gambiae* have been characterised during the last years (Lindh et al., 2015, Eneh et al., 2016). In the oviposition assays of this study we used distilled water to avoid any source of egg laying inhibition; however egg

retention occurs naturally in mosquitoes (Hitchcock, 1968, Magnarelli, 1975). Several factors have been linked to egg retention or oviposition inhibition; for example, *Culex pipiens fatigans* mosquitoes showed delayed oviposition when fed with cane sugar (de Meillon et al., 1967) and *Ae. albopictus* showed a higher egg retention rates when presented low moisture oviposition substrates (Saifur et al., 2010). Studies have reported natural retention rates of 4.5% in *An. quadrimaculatus* (n= 2983 parous mosquitoes) (Hitchcock, 1968), 20% in *An. punctipennis* (n= 49) (Magnarelli, 1975), with an egg retention of 1-2 eggs/mosquito only.

It is very important to understand the limitations of using egg retention as evidence of PPF action in the field: we cannot entirely rule out the possibility that some of the mosquitoes retaining eggs would have laid eggs after Day 6, the chosen time limit before dissections. Previous studies on *Ae. aegypti* used 7 days after bloodfeeding as 'forced egg retention' period (Chadee, 1997). We considered that after 6 days of bloodfeeding mosquitoes could start re-absorbing the eggs, making difficult to determine a PPF effect. Certainly some of the mosquitoes collected were under different physiological stages, and probably some of the gravid or even semi-gravid at the time of collection may have re-absorbed the eggs by the day of dissection. Other potentially important factors that was not addressed and could affect the egg retention rates were the mating status of the collected mosquitoes and the average number of gonotrophic cycles. It is expected than any of the mentioned effects could be equilibrated by the experimental design: mosquitoes collected before and after Duo were manipulated following strictly the same protocol.

The individual oviposition assays were an imperfect method of testing the effect of PPF, with moderate to low rates of egg laying, even in the mosquitoes exposed to untreated nets. This has also been observed by others: in an experimental hut trial with Duo in Ivory Coast the size of the oviposition batches ranged between 5 and 11 eggs per mosquito in either control or treatments, indicating flaws in their oviposition assay (Koffi et al., 2015), and in another trial of the same nature in Benin only 37% of control mosquitoes laid eggs (Ngufor et al., 2014). Instead, dissection and observation of the ovaries was found to be a

more reliable method to observe the impact of PPF exposure under laboratory conditions.

PPF has been already linked with egg retention in *An. arabiensis* (Harris et al., 2013). Although the author links egg retention with PPF action, they did not discriminate mosquitoes showing normal or abnormal ovaries. In the current study a morphological characterisation of the ovaries of mosquitoes retaining eggs provided evidence of a direct, observable effect of PPF rather than simply relying on egg laying. In a laboratory study, Koama *et al.* described the effect of PPF on oviposition and oogenesis in *An. gambiae.* In that study the oogenesis impairment caused by PPF was characterised, and it was suggested that the observed abnormalities caused by PPF were due to the unequal and slow development of ovary follicles (Koama et al., 2015).

5.4.2 Mosquitoes collected in houses with Olyset Duo lay smaller amounts of eggs

The average oviposition size was significantly reduced in mosquitoes that laid eggs after the distribution of Duo. This suggests that PPF can not only sterilise mosquitoes permanently, but when mosquitoes are able to develop and lay eggs successfully the amount of developed eggs is smaller than unexposed control mosquitoes. A possible explanation for this data is that some of the mosquitoes that laid eggs in the 'after Duo' arm did not acquire enough PPF to be sterilised, but partial sterilisation resulted in smaller egg batches.

A small scale field trial with Duo and PPF nets also found a significant reduction in the average number of eggs after an intervention with Duo (Kawada et al., 2014). In contrast an experimental trial in Ivory Coast failed to show any difference between the average egg batch size in interventions with Duo and untreated nets (Koffi et al., 2015); however the average number of eggs laid per mosquito was only 6 in the untreated control and 9 in Duo arms, suggesting some experimental limitations of the dataset. Further field trials with Olyset Duo are needed to further explore the impact on egg productivity in *An. gambiae* populations.

This results must be interpreted cautiously. First, the total number of mosquitoes laying eggs after the distribution of Duo were low compared to the

baseline arm of the study. Sample sizes before Duo ranged from 46 to 101, while after Duo it ranged from 5 to 15 individuals. Secondly, mosquitoes from the site control of Tengrela laid less than 76 eggs/mosquito, less than the overall mean of all the mosquitoes laying eggs after Duo. Third, all the collections for the 'after Duo' arm were done between September and October, and all the 'before Duo' collections were done in June-July. The exception was Bakaridjan, where collections took place exactly after one year of difference (September 2014 and 2015). Interestingly, this was the only village that did not show a significant difference in egg batch size between the time points. The body size of *Anopheles* can be correlated with the period of the climatic season: mosquitoes tend to be bigger during the dry season and smaller during the rainy season (Huestis et al., 2012). Bigger mosquitoes are expected to be able to obtain bigger bloodmeals leading to larger egg batches. A study done with anophelines from different regions from Burkina Faso showed a variable body size response to simulated 'rainy' and 'dry' season conditions; yet in Soumousso, a site located 87 km from Banfora district, *Anopheles* body size was significantly bigger during the rainy season (Hidalgo et al., 2015). In conclusion mosquitoes collected after Duo distribution showed a lower egg productivity than before the intervention, but this difference may be partially or completely due to sampling bias. A measure being currently considered to address this confounding factor is measuring wing length, which is used as an estimator of body size, and has been correlated with bloodmeal volume and egg productivity (Phasomkusolsil et al., 2015).

Another blind spot for the interpretation of this data, which is true for all the parameters measured, is that we cannot be certain that the mosquitoes laying eggs after the distribution of Duo were in contact with the actual LLINs, or simply obtained a bloodmeal without any exposure.

5.4.3 The progeny of mosquitoes collected in houses of Olyset Duo are less likely to hatch from eggs

Eggs laid by mosquitoes collected in Duo houses showed an overall diminished probability of hatching when compared with the baseline. This was also true for all the individual sites except for Sikane, where there was no significant

difference. As above, the effect on egg hatchability could be linked to sub-optimal exposures to PPF. Laboratory studies on the effect of PPF nets showed that egg hatchability was reduced in mosquitoes that were exposed to PPF at variable bloodfeeding regimes (Ohashi et al., 2012, Jaffer et al., 2015). The only small-scale field trial done with Duo showed no reduction in egg hatchability (Kawada et al., 2014), neither did the experimental hut trial in Benin (Djenontin et al., 2015). However, the experimental hut trial in Ivory Coast did show an effect of Duo on hatching rates (Koffi et al., 2015).

Reduced hatch rate has been linked with 7 days of oviposition-site deprivation in *An. gambiae* (Dieter et al., 2012). Although the mosquitoes in our experiments were not deprived of an oviposition surface, it is expected that a proportion of mosquitoes don't lay eggs in the artificial set up for behavioural reasons. In this way, delay in oviposition could hypothetically lead to reduced hatching rates (although this reduction would be expected to be the same between arms). Another important factor is that, although the majority of mosquitoes collected were recently bloodfed, some of them were half-gravid or even gravid. Therefore by the time of oviposition, the eggs could have been retained for a variable number of days.

The blood source can affect egg hatch rate. In *Anopheles sp.,* mosquitoes that were fed on sheep blood laid less eggs that also showed reduced hatchability compared with mosquitoes fed on human blood (Phasomkusolsil et al., 2013). Our characterisation of the blood source suggested high human blood rates but it also detected opportunistic feeding behaviour on animals.

In conclusion, we demonstrated a significant reduction in hatching rates of mosquitoes collected in houses with Duo LLINs. This coupled with the sterilising effect and reduced egg batch size suggest that Duo nets have a considerable impact in reducing the reproductive output of mosquitoes under field conditions.

5.4.4 Aspects and implications of the field trial

The current study is only the second study to measure the impact of Duo on mosquito reproductive under full field conditions. The earlier study was a small scale study involving 15 households and found an overall reduction in the reproductive output of mosquitoes (Kawada et al., 2014). The current study

involved collections from 286 households using Duo (i.e. not counting the households collected in the baseline). Mosquitoes were collected early in the morning, resting inside houses that had been using LLINs, but it was impossible to quantify the proportion that had actually contacted the LLINs or the duration of the mosquito-net contact. The persistence of the sterilisation effect after 12 months of Duo usage in Naniagara village is remarkable, but since only one datapoint was obtained further monitoring is ongoing in additional villages to confirm the results. The human blood index was high (although the sample size was low) but it is not known if bloodfed mosquitoes found inside houses had fed on humans sleeping under an LLIN or on unprotected individuals. Furthermore, as the parity rate was not measured, it was not possible to estimate the age of the population, or assess likelihood that females may have been exposed to PPF in previous gonotrophic cycles.

Although this study took place in the context of a clinical trial in which nets were freely distributed to cover all sleeping spaces (Tiono et al., 2015), usage of the nets was variable. The number of households using LLINs was particularly low in Tiefora Centre, which is a centre of commerce in the region. Compared with the other sentinel sites of this study it is more densely populated, and has facilities such as a health centre, shops, traditional bars and a school. The low use of nets in this village provided an opportunity to observe the potential community effect of Duo. Although the proportion of mosquitoes sterilised was higher in houses using Duo the previous night, 13 % of mosquitoes from houses with no bednets were also sterile. Presumably these mosquitoes had come into contact with an Duo when entering a separate house in search of a blood meal.

In conclusion, in this chapter we presented compelling evidence that Duo reduces different key factors of wild *An. gambiae* reproduction.

Chapter 6 Conclusions and next steps for the evaluation of Olyset Duo to control malaria mosquitoes

Pyriproxyfen is already being used to control mosquito larvae due to its potent inhibitory effects on metamorphosis but it is the impact of this chemical on adult mosquitoes that is potentially of even greater interest for malaria control. This study aimed to improve the knowledge on the effectiveness of PPF in controlling *Anopheles* mosquitoes under a range of experimental conditions in the laboratory and in the field. This chapter considers the evidence for the impact of exposure to PPF on critical aspects of a mosquito's life history (lifespan and reproductive output) and the impact of the first LLIN containing PPF, Olyset Duo, on the mosquito population in a large-scale field trial.

6.1 *Pyriproxyfen and Olyset Duo decreases mosquito lifespan*

In order to transmit the malaria parasite, mosquitoes must bite at least twice: one for acquiring the parasite, and the next one to transmit it to a human host. After a mosquito becomes infected by *Plasmodium spp.*, it takes between 8-15 days (depending on the parasite species and temperature) to become infective to humans (Clements, 1992). Thus reductions in the lifespan of adult female mosquitoes can have a dramatic impact on malaria transmission (Viana et al., 2016). In the longevity experiments with insecticide resistant/susceptible mosquitoes reported in Chapters 2 and 3 (sections 2.3.1 and 3.3.1 respectively), PPF exposure was shown to decrease the mosquito's lifespan by approximately 2-5 days in the laboratory. In experiments with pyrethroid-susceptible mosquitoes and a variety of bloodfeeding regimes, the magnitude of the PPF effect was greatest when mosquitoes were exposed to the chemical 24 hours after bloodfeeding. The hazard ratios for mosquitoes in the regimes that resembled certain real life scenarios (*i.e.* mosquitoes exposed to the net 24

hours before or immediately before bloodfeeding) showed that mosquitoes are 1.5 – 2 times more likely to die earlier than mosquitoes exposed to an untreated net. Experiments with Duo on multi-resistant laboratory and wild mosquitoes revealed a similar impact on mosquito longevity (section 3.3.1).

Although these results are promising, several limitations should be noted. First, these experiments were done under controlled laboratory conditions with colonised mosquitoes (with the exception of one group of wild mosquitoes from Naniagara village). The natural lifespan in the wild is likely to be considerably shorter than observed in the laboratory because factors like temperature, humidity, predation, pathogens, larval habitat, etc. are largely fluctuating (Maharaj, 2003, Okech et al., 2007, Yamana and Eltahir, 2013, Ng'habi et al., 2015). A potential alternative to evaluate the effect of Duo nets on mosquito longevity compared with controls in the field is the captive cohort method (Papadopoulos et al., 2016), which consist in collecting cohorts of individuals regularly followed by survival measurements. Second, the exposure time to the nets containing PPF was the standard 3 min recommended by the WHO (World Health Organization et al., 2013); now there is evidence that mosquitoes may be in contact with the nets for a much shorter time (Parker et al., 2015) and it is not known whether lifespan would also be affected by a short exposure. Third, the mechanism of lifespan reduction by PPF is unknown, so is not clear yet if all mosquito populations would be affected in a similar degree. In the only study addressing PPF impact on mosquito longevity, Ohashi *et al.* (Ohashi et al., 2012) showed a dose-dependent negative effect of PPF on mosquito longevity; however the magnitude of the effect was not discussed, so there is no point of reference to compare this study. In a study with the effect of the entomopathogenic fungus *Beauveria bassiana* on *An. gambiae* lifespan showed a survival hazard of 2.3, in a similar range to what was recorded in the present study (sections 2.3.1 and 3.3.1). In general, the effect of PPF on insect longevity is highly variable with effects reported in the literature varying between significant, drastic effects to no effect (Liu, 2003, Steigenga et al., 2006, Rugno et al., 2016).

Field and semi-field experiments including mark-release-recapture experiments could help measure the impact of PPF exposure on mosquito longevity under more natural settings. For example, mark-release experiments helped to characterise the longevity of an *An. funestus* population in the Kenyan coastline (Midega et al., 2007) and to obtain novel information of mosquito longevity during the dry season in the Sahel (Lehmann et al., 2010). However, although near infrared technology shows some promise (Sikulu et al., 2014, Liebman et al., 2015a), measuring mosquito age in very challenging. Indirect methods, such as measuring the parous rates of field caught mosquitoes as has been carried out in the context of the AVECNET Olyset Duo Controlled Trial, will hopefully prove informative (Tiono et al., 2015).

6.2 *Exposure to PPF and Olyset Duo sterilises Anopheles gambiae under laboratory conditions*

Experiments under laboratory conditions showed that 1% PPF nets permanently sterilise Kisumu strain susceptible mosquitoes after a 3 minute exposure in different bloodfeeding regimes (section 2.3.2). In one of the regimes, when mosquitoes bit the host freely through the PPF net, the sterilisation was not complete; however, since the time of exposure was not controlled a comparison with the other regimes was not possible. The PPF net was equally effective sterilising Tiassalé multi-resistant mosquitoes under a single exposure pre-bloodmeal regime (section 3.3.2). Studies reporting differential effects of PPF on mosquitoes depending on bloodfeeding regimes have caused debate (Ohashi et al., 2012, Harris et al., 2013, Koama et al., 2015). Since in this study no variation was observed within a range of 24 hours before and after bloodfeeding, those claims were not supported. The concentration in the PPF net used in this study (1%) exceeded too much the doses used in those studies (except in (Koama et al., 2015) were the PPF dose was the same) and therefore it could have masked any bloodfeeding-PPF exposure correlation.

The effect of PPF on mosquito sterilisation was dose-dependent (section 3.3.2). This has been shown previously by Ohashi *et al.* (Ohashi et al., 2012), who showed that 0.1% and 0.01% PPF nets sterilised completely mosquitoes, while

0.001% nets failed to do so. Our data provides the first range of PPF a.i. concentrations causing no, partial and complete sterilisation in anopheline mosquitoes (section 3.3.2).

The stability of PPF in this net (or Olyset Duo) under field conditions is unknown, but currently is being evaluated (Sagnon et al., 2015). The reports on the stability of PPF under field conditions are limited. Hargrove *et al.* described a loss of 60-85% of the total content of PPF impregnating cloth targets for the control of Tse tse flies during four months of deploying with a consequent loss of efficacy (Hargrove and Langley, 1993), and suggested that better formulations were needed.

6.3 *Effectiveness of pyriproxyfen in sterilising* Anopheles *when combined with permethrin in Olyset Duo*

Data from cone bioassays indicate that Duo LLINs are less effective at sterilising mosquitoes than nets containing PPF alone at the same concentration (section 3.3.2). Potential reasons for that are: 1) reduced bleed rate of PPF as a consequence of interaction with permethrin molecules that results in reduced PPF uptake by the mosquito; 2) The repellent effect of permethrin (Lindsay et al., 1991, Chandre et al., 2000) reduces the contact of the mosquito with the nets during the 3 minutes of the cone bioassay and 3) The presence of permethrin induces the production of enzymes that interact and neutralise PPF. Testing the first and second hypotheses was out of the scope of this study, and there is no enough evidence available to support them. Ngufor *et al.* affirms that PPF-only nets have a similar bleed to Duo nets, but there are no studies detailing the specific bleed rate of PPF in combination with permethrin. Similarly, there is not precise information to support the second hypothesis: there are no studies that directly explore the correlation between repellence and mortality by permethrin, or either explore the minimum time of contact between mosquito and net to deliver an effective dose of insecticide (Parker et al., 2015). To explore the third possibility a set of bioassays using PPF on resistant and susceptible mosquitoes were done (section 3.3.2).

Resistant mosquitoes showed a higher level of tolerance to PPF compared with susceptible in dose-response assays testing the pupicidal formulation's effect

on metamorphosis and the active ingredient on oogenesis. These results suggest a moderate level of cross-resistance. In vitro experiments using recombinant P450s indicate that several P450s that can metabolise permethrin, are also capable of metabolising PPF and the two chemicals act as competitive inhibitors of these P450s (Yunta et al., 2016). The laboratory assays in this study only compared a single susceptible and resistant population and therefore other strain-specific differences unrelated to their pyrethroid resistance status may be responsible for the observed difference in PPF/Olyset Duo efficacy. But this is not the only study to suggest a possible reduced performance of Duo in areas of high pyrethroid resistance: Koffi *et al.* reported no differences in fecundity of multi-resistant mosquitoes collected in huts with PPF-only, Duo or untreated nets (Koffi et al., 2015). However, as mentioned before, accuracy of those results could be limited by flaws in the oviposition assays.

Further experiments characterising the putative cross resistance between PPF and pyrethroids are critical to inform decisions about when and where Duo might be deployed. A standardised monitoring protocol for the detection of PPF resistance in adult mosquitoes should also be agreed upon. This is not straight forward, given the multiple different endpoints that could be assessed.

6.4 *Impact of Olyset Duo on wild multi-resistant mosquitoes under field conditions*

Under full field conditions, the impact of the introduction of Duo on mosquito reproductive output was very clear (section 5.4.1). Mosquitoes were collected from inside houses in villages using conventional LLINs and then collections were repeated in the same villages after the Olyset LLINs had been replaced with Duo LLINs. Comparing the reproductive outputs of the mosquito populations from the two different timepoints, and repeating this for 5 villages, enabled the conclusion that community scale use of Duo dramatically increased the number of sterile mosquitoes and reduced the reproductive output of those mosquitoes not fully sterilised. Unlike the laboratory assays, the field study was unable to directly assess exposure to LLINs. It is not known if mosquitoes collected inside houses with Olyset Duo nets have contacted the net in this, or any other household, prior to collection, nor is it possible to determine at what

point in the gonotrophic cycle the mosquitoes contacted the PPF. Despite this, the reductions in egg production were significant with the number of sterile mosquitoes reaching 64% in some villages (Figure 5.6).

Olyset Duo was distributed in the 5 sentinel villages 3-4 months before the second round of collections, so it was not possible to compare persistence of the sterilising or any other effect. That question is being currently addressed in laboratory assays with net samples from the field, and additional field mosquito collections to evaluate the sterilisation performance.

Although this study was done as part of a controlled trial, it was clear that not all households were using nets provided for the trial. This was particularly true for Tiefora Centre, where net usage was 54% only 4 months after the distribution of Duo. This is particularly concerning as it has been suggested that the rates of LLINs loss in African households are higher than previously thought (Bhatt et al., 2015b). However, this poor Duo coverage context provided the opportunity to examine the evidence for a community effect. As reported in section 5.3.4.1 (Figure 5.4), 13% of all mosquitoes collected in households with no bednets showed unequivocal signs of sterilisation by PPF. This suggests that even if the coverage with Duo is not global, mosquitoes could be in contact with those LLINs at some point in their life and become sterile. This hypothesis and the effect on population density are interesting topics to address in future studies.

6.5 *Permethrin resistance was reduced in different time exposures after the distribution of Olyset Duo under field conditions*

The results of this thesis suggest that Duo is a promising new tool for controlling pyrethroid resistant mosquito populations. Despite the possible presence of cross resistance between the two active ingredients in the Duo net (section 3.3.2), the field trials indicated an impressive reduction in the reproductive output of female mosquitoes after the introduction of Duo. Whether or not this (and/or other effects of PPF such as reduced adult lifespan) leads to a sufficient reduction the entomological inoculation rate to reduce malaria transmission will not be known until the results of the clinical trial are analysed later in the year.

A second potential beneficial impact of large-scale implementation of Duo would be a reduction in the prevalence and intensity of pyrethroid resistance. The

hypothesis is that pyrethroid resistant mosquitoes are sterilised by exposure to the PPF in the Duo LLINs, and therefore the resistant alleles will not be passed on to future generations. In chapter 4, the results of a very preliminary study to test this hypothesis were discussed. The standard WHO susceptibility tests, that failed to show any difference before and after Duo recommend to expose the mosquitoes for 60 minutes; however, given the unprecedentedly high levels of resistance this exposure time may be inadequate to measure significant changes over time. Permethrin exposures of 90 and 120 min showed a comparative decrease in resistance, suggesting that Duo can be working as hypothesised.

There are not many field trials testing insecticide resistance management strategies. One alternative is the use of insecticides with different modes of action in rotation or mixtures (Keiding, 1963); however, the success depends heavily on negative cross-resistance or resistance reversal, evidence for which is controversial (Kolaczinski and Curtis, 2004, Raghavendra et al., 2010). Since the only insecticides currently available for use in LLINs are pyrethroids, the only manner of doing rotations or mosaics with other insecticide classes is by implementing simultaneous indoor residual spraying (IRS).

A more convenient and reliable strategy to tackle insecticide resistance is by the use of nets combining insecticides and a non-insecticide. The combination of the synergist PBO with permethrin (Olyset Plus) and deltamethrin (Permanet 3) has proven more effective to kill resistant mosquitoes; however, some hyper-resistant populations can survive in high proportions to these nets already. The possibility of 'avoiding' the effect of PBO by increasing resistance mechanisms others than CYP450s is too real to ignore. The PPF in Duo does not confront the insecticide resistance mechanisms directly, but rather affects the reproductive output of the mosquitoes and reduces its lifespan (Ohashi et al., 2012, Ngufor et al., 2014, Tiono et al., 2015).

6.6 *Pyriproxyfen effectiveness in controlling other insect species suggest a good potential as a primary or complementary vector control tool*

PPF has been tested comprehensively in a number of formulations, methods, species and expected outcomes with success. PPF exposure was correlated

with a decrease in fecundity and hatch rates in *Ae. albopictus* (Ohba et al., 2013), and laboratory assays on *An. stephensi* found reduced egg hatching rates (Aiku et al., 2006). A study on *Ae. aegypti* using a fumigant formulation of PPF showed a dose-dependent hatch rate reduction (Harburguer et al., 2014), and a field trial suggested mosquito density decrease (Doud et al., 2014). Using Sugar toxic baits showed promising auto-dissemination of PPF by defecation in *Ae. albopictus* (Scott et al., 2016). The insecticidal paint Inesfly 5A®, containing two insecticides plus PPF, reduced the fecundity and fertility of *Culex quinquefasciatus* in laboratory experiments (Mosqueira et al., 2010b); however, they do not mention measurements of the impact on mosquito reproduction in their experimental huts trial publication (Mosqueira et al., 2010a). Also in laboratory tests, PPF affected negatively one or more reproductive trait in a range of holometabolous insects (Tassou and Schulz, 2009, Liu et al., 2012, Tay and Lee, 2014, Singh and Kumar, 2015, Xu et al., 2015). Field tests with *An. arabiensis* showed that PPF in grounded formulations is auto-disseminated successfully by mosquitoes (Lwetoijera et al., 2014a). All these studies are just part of a growing set of evidence supporting the use of PPF in the control of insects and pests.

6.7 *Future Work*

This thesis provided evidence of the effect of PPF and Olyset Duo on the reproduction and longevity of mosquitoes under laboratory and field conditions. It also standardised a series of experimental protocols that can be followed and improved for monitoring the effect of Duo under field conditions. More importantly, this thesis gave a preliminary view in how the effect of an insecticide resistance management intervention can be measured in terms of the strength of the insecticide resistance in wild mosquito populations.

Efforts on the characterisation of the mode of action of PPF should be done, as in its understanding relays PPF resistance prevention. To the date little is known about the mechanisms of action in which PPF reduces longevity or affects oogenesis. The moderate level of PPF tolerance shown by Tiassalé should be a warning, and other multi-resistant mosquito populations should also be tested and compared for the effects on metamorphosis and reproduction.

PPF nets having a bigger impact on mosquito lifespan and oogenesis than the combination with permethrin could be an opportunity to investigate alternatives in the design of LLINs. From a practical point of view it would be interesting to evaluate a LLIN that instead of mixing PPF and permethrin in all the panels, would have PPF-only on the roof and PPF plus permethrin in the side panels. Behaviour experiments on the interaction of *An. gambiae* with LLINs show that most of the mosquito activity is done on the roof (Parker et al., 2015). This approach is already used in Permanet 3 nets, that adds PBO and a higher concentration of deltamethrin in the roof panel (Tungu et al., 2010). It would be interesting to see if under this approach the strong impact on mosquito longevity and oogenesis shows any variation in relation to the PPF or Duo LLNIs.

Monitoring the insecticide resistance phenotype and mechanisms in Banfora district population is essential. Using either LT50 or LC50 to measure the strength of resistance throughout the coming years would provide valuable information on the potential efficiency of Duo in insecticide resistance management. There are other aspects to address under field conditions. The extent of the community effect of PPF could be examined by collecting mosquitoes in sentinel houses without LLINs and monitoring changes in the reproductive output. The behaviour of mosquitoes could also be followed through the intervention, aiming to observe any shift from endophilia to exophilia in the local populations. The durability of the LLNIs should be tested *in situ* to evaluate the effectiveness of the PPF under different periods of time.

A RCT in a different location is necessary to validate these results, as a single event cannot be generalised. This thesis plus the three experimental hut trial already published should constitute a solid baseline for the design and implementation of another full RCT.

Appendix

Table A1. Geographic information of the villages where mosquitoes were collected for the study. Tengrela village was selected as a negative control, without intervention.

Health Centre	Village	Coordinates
Tiefora	Tiefora Centre	10˚37’54.02’’N; 4˚33’22.85’’W
	Moussoumourou	10°35'48.93"N; 4°24'39.61"W
	Djomale	10˚33’17.24’’N; 4˚22’41.14’’W
	Pont Maurice	10˚38’26.71’’N; 4˚29’41.62’’W
	Sikane	10˚34’27.49’’N; 4˚22’38.16’’W
	Libora	10°34'9.54"N; 4°24'29.73"W
Kankounadeni	Naniagara II	10˚32’9.15’’N; 4˚40’7.84’’W
Koflande	Bakaridjan II	10˚24’26.34’’N; 4˚33’44.78’’W
	Koflande	10°10'8.36"N; 4°28'33.92"W
Madiasso	Bounouba	10°21'27.49"N; 4°26'20.07"W
Banfora regional hospital	Tengrela	10˚38’7.53’’N; 4˚48’48.35’’W

Table A2. Mortality in different villages of Banfora District in CDC bottle assays. All results are for bioassays in 2013 except for Tiefora 2015, as labelled in the table. Mortality percentages are given in percentages per concentration.

Village	Concentration	Sample size	Mortality %
Tiefora	5 ppm	92	16.3
	10 ppm	33	9.1
	20 ppm	76	31.6
	40 ppm	118	66.9
	60 ppm	28	71.4
	80 ppm	75	78.7
	100 ppm	11	100.0
	120 ppm	63	88.9
Kankounadeni	5 ppm	94	14.9
	10 ppm	24	8.3
	20 ppm	76	46.1
	40 ppm	106	73.6
	60 ppm	26	46.2
	80 ppm	49	98.0
	100 ppm	17	82.4
	120 ppm	32	100.0
Bakaridjan	5 ppm	62	12.9
	10 ppm	18	11.1
	20 ppm	41	73.2
	40 ppm	47	68.1
	60 ppm	17	76.5
	80 ppm	63	93.7
	100 ppm	16	100.0
	120 ppm	65	93.8
Naniagara	5 ppm	38	5.3
	10 ppm	13	0.0
	20 ppm	48	31.3
	40 ppm	31	58.1
	80 ppm	21	95.2
Bounouba	5 ppm	52	7.7
	20 ppm	41	36.6
	40 ppm	40	72.5
	80 ppm	42	83.3
	120 ppm	40	85.0
Tiefora 2015	5 ppm	97	2.1
	10 ppm	103	20.4
	20 ppm	100	58.0
	30 ppm	91	97.8
	50 ppm	102	97.1

Table A3 SINE PCR conditions for the species identification withing the *Anopheles gambiae* complex.

Mastermix component	**1x**
Water	17.33 µl
10x Buffer	2.5 µl
dNTP (10mM)	0.5 µl
6.1a	1 µl
6.1b	1
MgCL2 (25 mM)	1.5 µl
Taq	0.17 µl
Template DNA	**1 µL**
Final volume	2 µl

Conditions	
Denaturation	95°C - 5 minutes
35 cycles	95°C - 30 seconds
	54°C - 30 seconds
	72°C - 60 seconds
Final elongation	72°C - 10 minutes

Table A4 Species abundance from larval collections in three villages of Banfora district.

Village	Year	n	An. coluzzii	An. gambiae ss	An. arabiensis
Tiefora	2013	40	65%	25%	10%
	2014	98	6.0%	93%	1%
	2015	40	5%	90%	5%
Naniagara	2013	19	5%	84%	11%
	2014	86	14%	86%	0
	2015	45	9%	84%	7%
Bakaridjan	2013	36	34%	60%	6%
	2014	95	5%	90%	5%
	2015	42	5%	90%	5%

Table A5 Mortality after exposure to a range of conventional or combination nets of mosquitoes from different villages from the study site by WHO cone bioassays. All

mosquitoes were raised from larval collections, except Naniagara F1 that came from ovipositions of mosquitoes collected indoors.

Village	Net type	Total	Percentage mortality	Confidence interval 95%
Tiefora	Olyset	95	7.4	3.3 – 15.1 %
	Olyset Plus	105	26.7	18.7 – 36.3 %
	Olyset Duo	84	14.9	8.7 – 24.1 %
	Permanet 2	101	4.0	1.3 – 10.4 %
	Permanet 3 (side)	96	42.7	32.8 – 53.2 %
	Permanet 3 (roof)	99	75.8	65.9 – 83.6 %
Naniagara	Olyset	101	10.9	5.8 – 19 %
	Olyset Plus	105	22.9	15.5 – 31.8 %
	Olyset Duo	265	32.8	27.3 – 38.9 %
	Permanet 2	100	30.0	21.4 – 40.1 %
	Permanet 3 (side)	103	46.6	36.8 – 56.7 %
	Permanet 3 (top)	94	74.5	64.2 – 82.7 %
Bakaridjan	Olyset	98	1.0	0.0005 – 6.4 %
	Olyset Duo	105	9.5	4.9 – 17.2 %

Table A6. Kdr frequency through 2013-2015. Fisher's exact test for the variability of kdr allelic frequencies in 2013 – 2015 for *An. gambiae* and *An. coluzzii.* No significant difference was found for any comparison.

		2013-2014	2014-2015	2013-2015
Mutation	**Species**	**p-value**	**p-value**	**p-value**
L1014F	An. gambiae	0.11	0.27	0.68
	An. coluzzii	0.594	0.72	0.48
N118Y	An. gambiae	0.14	0.82	0.23
	An. coluzzii	0.4	0.43	1

Table A7. Enrichment analysis of overexpressed and underexpressed transcripts. Percentages of enriched annotation terms in the lists of significantly overexpressed and underexpressed transcripts from each microarray comparing: Tiefora 2013 vs Kisumu (Ti2013), Banfora S vs Kisumu (BanS) and BanforaM vs Ngousso (BanM).

	Overexpressed					
	Ti2013		BanS		BanM	
Term	%	p Value	%	p Value	%	p Value
Secondary metabolites biosynthesis, transport, and catabolism	42.4	2.25E-29	43.8	3.16E-28	43.1	1.76E-15
Metabolism of xenobiotics by cytochrome P450	11.1	2.74E-16	11.2	1.79E-15	9.8	2.34E-06
Drug metabolism - cytochrome P450	11.1	2.74E-16	11.2	1.79E-15	9.8	2.34E-06
Glutathione metabolism	12.1	9.95E-14	11.2	9.95E-12	11.8	2.15E-06
Lipid metabolism	11.1	0.001	7.9	0.07	9.8	0.07
Peroxisome	5.1	0.005	4.5	0.01	5.9	0.05
Insect hormone biosynthesis	3.0	0.01				
domain:GST N-terminal	2.0	0.03				
domain:GST C-terminal	2.0	0.03				
Posttranslational modification, protein turnover, chaperones	11.1	0.09	13.5	0.018		
	Underexpressed					
	Ti2013		BanS		BanM	
Term	%	p Value	%	p Value	%	p Value
Secondary metabolites biosynthesis, transport, and catabolism	34.2	4.02E-08	35	1.15E-08	40	8.10E-12
Posttranslational modification, protein turnover, chaperones	21.1	0.003				
Glutathione metabolism	7.9	0.007				
Drug metabolism - cytochrome P450	5.3	0.057				
Metabolism of xenobiotics by cytochrome P450	5.3	0.057				
Insect hormone biosynthesis					6.7	0.001
Lipid metabolism					13.3	0.01

Table A8. Lists of the 20 genes with the highest fold change (FC) per microarray. Significance cut-off for all the microarrays was p<0.01 except for BanS vs Ti2013 (p<0.05). Annotated genes are in bold.

	Upregulated			Downregulated		
Microarray	**Name**	**Adj. p value**	**FC**	**Name**	**Adj. p value**	**FC**
Ti2013 vs Kisumu	AGAP006879	6.77E-05	57.3	AGAP006414	3.75E-06	0.03
	AGAP003778	3.76E-06	50.2	**CLIPB17**	3.76E-06	0.04
	AGAP004161	6.09E-06	48.4	AGAP013202	1.03E-05	0.05
	AGAP008438	3.76E-06	42.8	AGAP003713	4.36E-06	0.07
	AGAP003776	3.76E-06	29.5	AGAP007747	3.76E-06	0.08
	GSTS1	3.76E-06	29.5	AGAP002643	3.76E-06	0.08
	CPR75	1.27E-05	27.7	AGAP004880	3.75E-06	0.09
	AGAP011515	2.61E-05	27.0	AGAP005942	9.73E-06	0.09
	AGAP003773	7.17E-06	25.1	**PGRPS3**	8.64E-06	0.09
	AGAP003777	5.21E-06	22.8	AGAP000152	4.36E-06	0.09
	AGAP002358	8.98E-05	21.9	AGAP001819	2.68E-05	0.1
	AGAP011460	1.90E-05	21.3	AGAP007959	5.46E-05	0.11
	SP11372	1.23E-05	20.4	AGAP012107	2.10E-05	0.11
	AGAP004437	1.24E-05	20.1	AGAP003635	1.07E-05	0.11
	AGAP003775	6.45E-06	19.7	AGAP009998	2.52E-04	0.11
	AGAP013256	3.76E-06	17.8	AGAP007053	7.69E-06	0.11
	AGAP003939	2.78E-05	16.8	**RpS11**	3.30E-03	0.12
	AGAP013005	8.17E-06	16.7	AGAP011938	4.56E-06	0.12
	AGAP011930	4.16E-06	16.4	AGAP009049	1.17E-05	0.13
	AGAP001594	3.75E-06	16.0	AGAP007365	3.64E-04	0.14
BanS vs Kisumu	**SP11372**	3.39E-04	47.2	**CEC1**	3.25E-04	0.01
	AGAP006879	7.89E-04	46.2	AGAP002643	3.39E-04	0.03
	AGAP012201	3.39E-04	41.6	AGAP006710	3.93E-03	0.04
	AGAP008449	4.02E-04	40.7	AGAP013202	3.39E-04	0.04
	AGAP004690	1.60E-03	31.1	AGAP005942	4.29E-04	0.04
	AGAP004161	8.41E-04	31.0	AGAP009049	4.20E-04	0.05
	CPF3	5.97E-04	26.1	AGAP009998	3.25E-04	0.05
	AGAP008450	3.39E-04	25.9	AGAP012296	3.25E-04	0.06
	CHYM1	5.98E-04	25.8	AGAP007747	3.39E-04	0.06
	AGAP003778	4.65E-04	25.6	AGAP001582	3.39E-04	0.06
	AGAP008444	3.55E-04	24.8	**PGRPS3**	3.39E-04	0.06
	CPCFC1	4.28E-04	24.4	AGAP006342	1.38E-03	0.07
	D7L2	4.20E-04	23.8	AGAP000693	3.25E-04	0.07
	D7r2	3.39E-04	22.7	AGAP003635	7.52E-04	0.07
	CPLCG1	4.67E-04	21.9	AGAP012359	3.85E-04	0.07
	CPLCG4	8.65E-04	21.6	**RpS11**	4.11E-04	0.08
	CPLCG5	5.80E-04	19.7	**CLIPB14**	3.85E-04	0.08
	AGAP008282	7.93E-04	19.1	**CYP9J5**	3.25E-04	0.08

	CPR75	4.04E-04	18.4	AGAP004880	3.92E-04	0.08
	AGAP006709	4.20E-04	17.6	AGAP002359	3.39E-04	0.09
	Upregulated			**Downregulated**		
Microarray	**Name**	**Adj. p value**	**FC**	**Name**	**Adj. p value**	**FC**
BanM vs Ngousso	AGAP006710	4.69E-04	148.1	AGAP006414	3.35E-04	0.02
	AGAP004146	4.09E-04	56.4	AGAP007039	6.58E-04	0.03
	AGAP007386	3.35E-04	46.5	AGAP007053	8.03E-04	0.04
	AGAP006400	1.60E-04	42.7	AGAP006181	1.25E-03	0.04
	AGAP003968	2.47E-03	38.0	**A5R1**	4.03E-04	0.05
	AGAP004400	3.35E-04	37.8	AGAP006177	7.26E-04	0.05
	AGAP002878	6.24E-04	33.2	**CHYM2**	1.26E-03	0.05
	AGAP007160	5.57E-04	31.2	**REL2**	8.79E-04	0.06
	AGAP006709	4.69E-04	26.1	AGAP007050	1.26E-03	0.06
	AGAP006504	3.35E-04	21.4	AGAP005822	2.81E-03	0.07
	AGAP008438	6.59E-03	20.4	**LRIM4**	1.22E-03	0.07
	SG2b	2.00E-03	19.8	AGAP012012	8.24E-04	0.08
	CHYM1	8.91E-04	19.3	AGAP010363	1.96E-03	0.10
	AGAP000618	3.35E-04	18.7	AGAP007049	1.30E-03	0.10
	AGAP001239	3.35E-04	18.2	AGAP006778	7.12E-04	0.10
	AGAP012129	3.35E-04	18.0	AGAP007064	7.82E-04	0.10
	LYSC7	7.58E-04	17.6	AGAP011515	1.61E-03	0.10
	AGAP003473	1.25E-03	17.2	AGAP011460	1.08E-03	0.11
	AGAP006199	6.24E-04	17.2	AGAP006441	6.91E-04	0.11
	AGAP006365	4.69E-04	15.1	AGAP006747	4.69E-04	0.11
BanS vs Ti2013	**GAM1**	7.57E-03	20.2	**CPLCG5**	1.16E-02	0.04
	AGAP011305	1.48E-02	15.1	**LRIM8B**	1.21E-02	0.06
	AGAP009948	1.21E-02	8.2	**CPF3**	1.07E-02	0.06
	AGAP000082	1.21E-02	7.3	**CPLCG4**	1.16E-02	0.07
	AGAP006414	1.48E-02	6.8	AGAP012201	1.07E-02	0.08
	AGAP001508	4.53E-02	6.7	**CPCFC1**	1.23E-02	0.10
	AGAP010363	1.48E-02	5.9	AGAP008450	1.16E-02	0.11
	CEC1	1.16E-02	5.3	**CPR59**	1.16E-02	0.11
	AGAP007990	1.07E-02	5.3	AGAP008369	1.16E-02	0.12
	AGAP008922	1.43E-02	5.2	**CPLCA3**	1.96E-02	0.12
	AGAP002630	1.32E-02	4.9	**CPLCX2**	1.07E-02	0.16
	LYSC2	1.43E-02	4.8	AGAP006584	1.07E-02	0.18
	AGAP002889	1.34E-02	4.8	AGAP007365	1.65E-02	0.18
	CYP6M1	2.20E-02	4.8	AGAP004031	1.65E-02	0.20
	AGAP003939	1.69E-02	4.6	AGAP006581	1.07E-02	0.20
	AGAP002878	1.54E-02	4.6	AGAP005310	1.34E-02	0.20
	TEP1	1.16E-02	4.4	AGAP005065	3.34E-02	0.20
	AGAP000529	1.07E-02	4.3	AGAP001065	1.50E-02	0.21
	AGAP005079	1.16E-02	4.2	**CPR131**	1.94E-02	0.21
	AGAP010911	1.55E-02	4.2	AGAP004802	1.29E-02	0.22

Table A9. Fold change of genes that were upregulated and downregulated simultaneously in the microarrays comparing resistant with susceptible mosquitoes: Ti2013 vs Kisumu (Ti2013), BanS vs Kisumu (BanS) and banM vs Ngousso (BanM).

	Upregulated				Downregulated		
Gene ID (name)	Ti2013	BanS	BanM	Gene ID (name)	Ti2013	BanS	BanM
AGAP006364 (ABCB4)	2.2	2.2	2.3	AGAP002203 (CYP325D2)	0.7	0.6	0.5
AGAP002417 (CYP4AR1)	6.6	5.0	3.8	AGAP008214 (CYP6M4)	0.4	0.5	0.5
AGAP009246 (CYP4C27)	3.1	5.0	2.3	AGAP000448	0.5	0.4	0.6
AGAP002418 (CYP4D15)	4.8	3.5	2.9	AGAP000693 (CEC1)	0.2	0.1	0.3
AGAP001076 (CYP4G16)	9.8	8.3	1.9	AGAP001198	0.3	0.5	0.1
AGAP002865 (CYP6P3)	3.4	2.9	3.4	AGAP002294	0.3	0.2	0.6
AGAP007480 (CYP6AH1)	1.6	1.7	4.4	AGAP002333	0.7	0.6	0.5
AGAP008218 (CYP6Z2)	4.4	3.6	2.6	AGAP002402	0.7	0.4	0.3
AGAP009946 (GSTMS3)	3.9	3.0	4.4	AGAP002969	0.4	0.5	0.5
AGAP000288	2.2	2.2	2.6	AGAP003141	0.4	0.3	0.5
AGAP000468	4.4	4.2	6.5	AGAP003142	0.4	0.7	0.2
AGAP000987 (CPAP3-A1b)	8.3	5.9	3.1	AGAP003400	0.5	0.2	0.5
AGAP001023	2.1	1.6	1.8	AGAP003485	0.3	0.3	0.4
AGAP001053	2.8	2.7	2.0	AGAP003635	0.1	0.1	0.2
AGAP001549	11.3	12.4	10.8	AGAP003713	0.1	0.1	0.3
AGAP001569	3.3	3.2	2.0	AGAP003823	0.4	0.4	0.4
AGAP001729	1.9	2.9	3.2	AGAP003934	0.5	0.5	0.5
AGAP001797	1.4	1.8	1.8	AGAP004018	0.5	0.5	0.6
AGAP001799	3.3	4.8	3.5	AGAP004199	0.3	0.2	0.2
AGAP001942	2.7	4.8	2.9	AGAP004310	0.5	0.5	0.4
AGAP001956	3.6	3.2	6.1	AGAP004440	0.6	0.6	0.6
AGAP001974	1.8	2.0	2.2	AGAP004684	0.2	0.2	0.6
AGAP001998 (mRpS10)	1.4	2.4	5.3	AGAP004880	0.1	0.1	0.2
AGAP002015	10.1	3.7	2.5	AGAP004987	0.6	0.3	0.5
AGAP002058	2.8	3.6	2.7	AGAP005160	0.4	0.5	0.2
AGAP002194	2.5	3.9	5.3	AGAP005796	0.7	0.6	0.3
AGAP002239	2.6	4.5	3.8	AGAP005822	0.3	0.2	0.1
AGAP002317 (Alpha_amylase)	14.9	14.0	3.2	AGAP005845	0.4	0.2	0.2
AGAP002324	2.8	3.5	2.4	AGAP005848	0.2	0.2	0.2
AGAP002364	8.0	8.8	4.0	AGAP005891	0.5	0.5	0.4
AGAP002377	1.5	1.7	2.1	AGAP005928	0.6	0.4	0.2
AGAP002505	2.1	1.4	2.7	AGAP005963	0.6	0.4	0.5
AGAP002603	2.8	3.3	4.2	AGAP006022	0.7	0.7	0.2

				(Bhlh_PAS)			
AGAP002736	2.3	1.5	2.8	AGAP006070	0.3	0.1	0.1
AGAP003083	2.2	3.9	3.6	AGAP006206	0.3	0.2	0.3
AGAP003124	2.2	1.5	2.9	AGAP006235	0.4	0.6	0.2
AGAP003195	4.0	2.9	1.9	AGAP006250	0.4	0.4	0.3
AGAP003261	8.1	10.3	4.7	AGAP006398	0.2	0.3	0.2
AGAP003357	7.5	5.0	8.2	AGAP006414	0.0	0.0	0.0
AGAP003367	6.1	6.8	11.4	AGAP006421 (A5R1)	0.2	0.3	0.1
AGAP003371	4.6	2.6	5.0	AGAP006441	0.4	0.4	0.1
AGAP003422	2.2	3.0	2.5	AGAP006460	0.6	0.4	0.5
AGAP003499	2.9	2.9	1.8	AGAP006495	0.2	0.2	0.2
AGAP003502 (HPX6)	3.8	6.3	2.6	AGAP006543	0.5	0.3	0.3
AGAP003661	2.5	2.5	2.4	AGAP006579	0.4	0.2	0.4
AGAP003686	1.7	2.6	2.4	AGAP006652	0.4	0.6	0.4
AGAP003785	3.3	4.8	2.2	AGAP006747 (REL2)	0.4	0.2	0.1
AGAP004400	4.2	3.1	37.8	AGAP006757	0.4	0.3	0.2
AGAP004690 (CPF3)	2.2	31.1	7.9	AGAP006778	0.2	0.1	0.1
AGAP004709	2.0	1.7	2.2	AGAP006782	0.2	0.2	0.0
AGAP004771	3.2	2.5	2.7	AGAP006793	0.2	0.1	0.2
AGAP005061 (RpS9)	8.9	16.9	13.0	AGAP006815	0.4	0.5	0.4
AGAP005234 (CuSOD2)	6.0	5.0	1.8	AGAP006885	0.3	0.3	0.1
AGAP005289	10.9	12.0	1.8	AGAP006925	0.7	0.6	0.4
AGAP005313	3.6	5.7	5.3	AGAP006933	0.7	0.6	0.4
AGAP005323	3.6	3.4	4.3	AGAP006937	0.5	0.6	0.4
AGAP005327	3.6	4.8	2.0	AGAP006946	0.6	0.3	0.1
AGAP005528	2.0	2.8	2.6	AGAP006948	0.6	0.4	0.3
AGAP005750	5.8	14.7	2.2	AGAP006963	0.7	0.6	0.2
AGAP006186	2.4	2.5	3.7	AGAP006989	0.5	0.5	0.5
AGAP006260	1.9	1.9	2.5	AGAP006994	0.5	0.4	0.3
AGAP006501	2.3	4.3	3.0	AGAP007021	0.5	0.2	0.2
AGAP006504 (SG2b)	15.0	15.6	21.4	AGAP007049	0.4	0.4	0.1
AGAP006584	1.6	9.3	6.3	AGAP007053	0.1	0.1	0.0
AGAP006614	1.8	1.5	2.3	AGAP007064	0.2	0.3	0.1
AGAP006709 (CHYM1)	5.4	17.6	26.1	AGAP007747	0.1	0.1	0.1
AGAP006829 (CPR59)	1.7	9.8	3.9	AGAP007858	0.4	0.2	0.3
AGAP006879	57.3	46.2	19.8	AGAP008244	0.4	0.2	0.5
AGAP006898	5.1	4.0	3.9	AGAP008879	0.3	0.4	0.5
AGAP007042 (CPR62)	1.3	3.9	2.8	AGAP009146	0.4	0.3	0.5
AGAP007104	1.8	2.6	3.3	AGAP009264	0.5	0.3	0.5
AGAP007160	9.4	4.4	31.2	AGAP009365	0.6	0.5	0.4
AGAP007161	4.9	3.1	7.8	AGAP009377	0.6	0.6	0.4

AGAP007249 (Flightin)	6.2	4.6	6.7	AGAP009404	0.8	0.7	0.5
AGAP007484	1.6	3.0	2.4	AGAP009701	0.5	0.6	0.6
AGAP007963	2.4	3.5	2.2	AGAP009928	0.7	0.6	0.7
AGAP007980 (CPCFC1)	3.1	19.7	5.5	AGAP011323	0.5	0.6	0.5
AGAP008182	3.7	4.1	1.7	AGAP011951 (CASPS4)	0.5	0.3	0.4
AGAP008369	4.0	16.7	4.6	AGAP011981	0.5	0.3	0.4
AGAP008371	3.5	6.3	3.7	AGAP012103	0.5	0.5	0.6
AGAP008447 (CPLCG4)	4.5	26.1	11.8	AGAP012359	0.2	0.1	0.4
AGAP008449 (CPLCG5)	5.3	40.7	11.3	AGAP013419	0.4	0.3	0.5
AGAP008761	1.6	2.0	3.8				
AGAP008782	3.2	3.9	6.2				
AGAP008987	1.6	1.8	1.8				
AGAP009110	5.6	5.9	2.4				
AGAP009330	1.9	2.3	2.0				
AGAP009752	5.2	6.3	2.5				
AGAP009790 (CPAP3-B)	3.9	6.8	2.9				
AGAP009824	3.6	2.8	2.1				
AGAP009842	4.3	7.9	8.1				
AGAP009868 (CPR73)	1.5	3.1	2.0				
AGAP009871 (CPR75)	27.7	23.8	10.0				
AGAP010014	3.3	6.6	2.8				
AGAP010205	4.5	4.7	4.1				
AGAP010326	5.9	4.8	7.3				
AGAP010854	1.6	3.2	2.2				
AGAP010878	3.7	3.2	1.7				
AGAP011251	2.7	4.9	5.0				
AGAP011330	2.0	1.9	4.3				
AGAP011516	3.0	5.0	3.2				
AGAP011834	2.2	2.8	2.4				
AGAP012030 (mRpL21)	1.6	2.1	2.3				
AGAP012036	3.2	3.9	3.5				
AGAP012396	1.7	2.4	1.9				
AGAP012604	1.9	2.1	2.7				
AGAP012609	2.4	4.8	2.0				
AGAP012757	3.0	9.2	2.0				
AGAP012984	1.9	3.2	3.3				
AGAP013008	2.1	2.0	2.7				
AGAP013061	8.2	5.9	2.5				
AGAP013223	1.9	1.7	3.2				
AGAP013365	2.4	4.0	10.9				
AGAP013493	2.8	3.8	4.2				

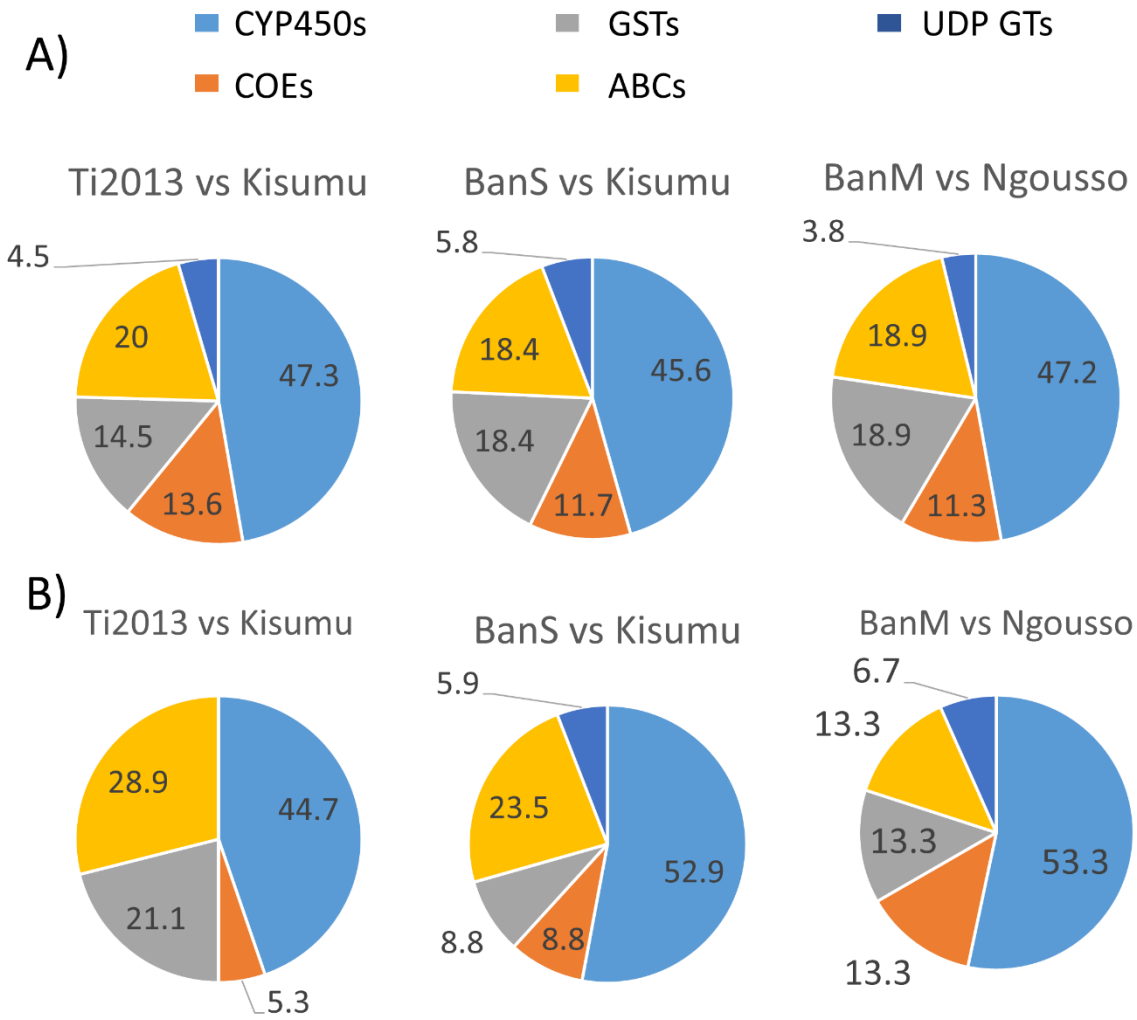
Table A10. Physiological status of the female Anopheline mosquitoes collected by indoor aspiration before and after the distribution of Olyset Duo in five sentinel sites of Banfora district.

Village	Intervention	Bloodfed	Gravid	No bloodfed	Total
Naniagara	Before Duo	ND	ND	ND	
	After Duo	242	22	27	291
Bakaridjan	Before Duo	ND	ND	ND	
	After Duo	156	21	7	184
Pont Maurice	Before Duo	211	0	2	213
	After Duo	164	13	43	220
Djomale	Before Duo	341	0	20	361
	After Duo	94	6	17	117
Sikane	Before Duo	236	0	3	239
	After Duo	184	3	8	195

Table A11. Mortality and number of mosquitoes that laid eggs during the oviposition assays and results of the ovary dissections.

BEFORE DUO	Dead	Mosquitoes laying eggs	Dissected mosquitoes unidentified	Dissected mosquitoes undeveloped ovaries	Dissected mosquitoes with ovaries scored
Naniagara	4	51	10	6	21
Bakaridjan	18	46	3	45	90
Pont Maurice	104	88	2	0	19
Djomale	242	99	1	1	18
Sikane	104	101	1	0	33
TOTAL	472	385	17	52	181
AFTER DUO					
Naniagara	90	7	14	4	176
Bakaridjan	31	12	8	19	114
Pont Maurice	104	15	6	3	92
Djomale	12	5	7	8	85
Sikane	56	15	1	13	110
TOTAL	293	54	36	47	577

Figure A1. Percentage of A) upregulated and B) downregulated detoxification gene families for each microarray comparing resistant and susceptible mosquitoes.



File A1. Publication in Malaria Journal as first co-author. This publication include the results of some experiments done in Chapter 4, aimed to improve the methodology for the monitoring of insecticide resistance.

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When a discriminating dose assay is not enough: measuring the intensity of insecticide resistance in malaria vectors

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Abstract

Background: Guidelines from the World Health Organization for monitoring insecticide resistance in disease vectors recommend exposing insects to a predetermined discriminating dose of insecticide and recording the percentage mortality in the population. This standardized methodology has been widely adopted for malaria vectors and has provided valuable data on the spread and prevalence of resistance. However, understanding the potential impact of this resistance on malaria control requires a more quantitative measure of the strength or intensity of this resistance.

Methods: Bioassays were adapted to quantify the level of resistance to permethrin in laboratory colonies and field populations of *Anopheles gambiae sensu lato*. WHO susceptibility tube assays were used to produce data on mortality versus exposure time and CDC bottle bioassays were used to generate dose response data sets. A modified version of the CDC bottle bioassay, known as the Resistance Intensity Rapid Diagnostic Test (I-RDT), was also used to measure the knockdown and mortality after exposure to different multipliers of the diagnostic dose. Finally cone bioassays were used to assess mortality after exposure to insecticide treated nets.

Results: The time response assays were simple to perform but not suitable for highly resistant populations. After initial problems with stability of insecticide and bottle washing were resolved, the CDC bottle bioassay provided a reproducible, quantitative measure of resistance but there were challenges performing this under field conditions. The I-RDT was simple to perform and interpret although the end point selected (immediate knockdown versus 24 h mortality) could dramatically affect the interpretation of the data. The utility of the cone bioassays was dependent on net type and thus appropriate controls are needed to interpret the operational significance of these data sets.

Conclusions: Incorporating quantitative measures of resistance strength, and utilizing bioassays with field doses of insecticides, will help interpret the possible impact of resistance on vector control activities. Each method tested had different benefits and challenges and agreement on a common methodology would be beneficial so that data are generated in a standardized format. This type of quantitative data are an important prerequisite to linking resistance strength to epidemiological outcomes.

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Background

Insecticides are a vital part of the malaria vector control tool box. In sub-Saharan Africa, where the vast majority of malaria morbidity and mortality occurs, the World Health Organization (WHO) recommends universal coverage with long-lasting insecticide nets (LLINs) treated with pyrethroids to reduce malaria transmission. Pyrethroids, plus also DDT, organophosphates and carbamates, are also being used in Indoor Residual Spraying (IRS) programmes in many African countries. The scale-up in coverage with LLINs and IRS have imposed a massive selection pressure on the malaria mosquitoes resulting in an escalation in insecticide resistance. This is compounded by the use of the same classes of chemicals in agriculture and, for pyrethroids, also in consumer products such as aerosols and coils [1].

Pyrethroid resistance was first detected in the two major malaria vectors *Anopheles gambiae s.l.* and *Anopheles funestus* in a small number of sites at the end of the last century [2–4]. The 21st century has witnessed a rapid spread of this resistance phenotype across the continent and it is now difficult to find sites in Africa where both vectors remain fully susceptible to pyrethroids [5]. Several different resistance mechanisms have been detected. Single amino acid substitutions at codon 1014 of the pyrethroid target site, the voltage gated sodium channel (known as *kdr* mutations), were the first mechanisms to be molecularly characterized [6, 7]. Further target site mutations have now been reported [8] in addition to potentially more potent metabolic and/or penetration based mechanisms [9, 10].

The vast majority of resistance monitoring in malaria vectors follows WHO protocols, revised in 2013, which recommend the use of susceptibility tube bioassays with papers coated with 'discriminatory doses' of insecticide [11]. Data are reported as percentage mortality and a threshold of less than 90 % mortality is used to define resistance (and mortality between 90 and 98 % is defined as suggestive of the presence of resistance). This standardized methodology is useful for tracking the spread of resistance but does not provide information on the strength of this resistance or its impact. The concentration of insecticide used has no relationship to the quantity of insecticide used in field applications but is instead set as twice the concentration required to kill a susceptible strain of the same species. Furthermore, by using prevalence of resistance as the metric, it is not possible to identify regions where resistance is likely to be posing the greatest threat to malaria control. Mosquitoes collected from site A may yield 50 % mortality in a discriminating dose assay whereas mosquitoes from site B may have been just under the threshold with 85 % mortality. This does not however mean that resistance is less of a threat in site B. The 15 % that did survive may have an extremely high level of resistance enabling them to readily survive long periods of time on a treated surface and thus potentially transmit malaria despite high coverage with vector control. In contrast, if the 50 % that survived the discriminating dose in site A have a relatively weak phenotype they will be killed when exposed to field concentrations of insecticide and thus pose less of an immediate threat. Data from Burkina Faso further illustrate how simply collating data on the prevalence of resistance can mask important changes in the strength of this resistance. Three years of monitoring insecticide resistance in *An. gambiae* from Vallée du Kou, in Southwest Burkina Faso using discriminating dose assays showed no significant difference in percentage mortality between the years but when a more quantitative measure was used to assess the strength of this phenotype, resistance was found to have increased ten -fold in a single year [12].

With no new insecticides expected to be licensed for use in malaria control before the end of the decade at the earliest, programmes need to make difficult decisions when faced with growing reports of resistance. Ideally a resistance management programme would be proactive rather than reactive but with only one insecticide class licensed for use on bed nets, and alternatives to pyrethroids frequently incurring higher costs for IRS, in reality, evidence of control failure is likely to be the only trigger for a change in insecticide policy. However, rather than waiting for insecticide failure to result in more deaths, it must be possible to re-define the way in which resistance is measured in the field to identify an 'operationally significant' threshold of resistance above which the gains from use of this insecticide class are lost. A necessary first step in this process is the development of simple bioassays that can measure resistance intensity so that resistance can be stratified according to the threat of control failure.

In this study, a variety of quantitative bioassays were used to assess the level of resistance in two laboratory strains and a field population of *An. gambiae s.l.* The consistency between the different assays and the relative ease of performing each method in the field were compared and the requirements for a reliable method that could be readily adopted under field conditions are discussed.

Methods

Mosquito strains

Two pyrethroid resistant laboratory strains of *An. gambiae s.l.* were used in the study with data from the insecticide susceptible Kisumu strain being used as a comparator. The Tiassalé strain was colonized from Southern Côte d'Ivoire in 2013 and maintained at the Liverpool School of Tropical Medicine (LSTM) under six-monthly selection pressure with deltamethrin. This strain, which contains

both *An. gambiae s.s.* and *Anopheles coluzzii*, is resistant to all four classes of insecticide currently available for malaria control [13]. The Tororo strain of *An. gambiae s.s.* was colonized from Eastern Uganda in 2013 and maintained at LSTM without selection pressure. In addition to the pyrethroid resistance described in this report, this strain is resistant to bendiocarb (65 % mortality after 1 h exposure to 0.1 % papers) and DDT (8 % mortality after exposure to 4 % papers).

Bioassays on wild caught mosquitoes were performed between May and September, 2014, on *An. gambiae* adults raised from larval collections from Tiefora, Banfora District, Burkina Faso (GPS coordinates: 10;37;54.02, 04;33;22.85). Both *An. gambiae s.s.* and *An. coluzzii* are found sympatrically in this site with *An. gambiae* predominating (63 % $n = 168$, June-September 2014).

All bioassays were performed on 3–5 days old, non blood-fed females. Bioassays on the laboratory colonies were performed in the insectaries at LSTM. Assays on the Tiefora population were performed at the Centre National de Recherche et de Formation sur le Paludisme (CNRFP) insectaries in Banfora. In all bioassays mosquitoes were considered dead when they couldn't stand or fly in a coordinated way.

WHO susceptibility assays

WHO susceptibility tests were performed using papers obtained from Universiti Sains Malaysia, impregnated with 0.75 % permethrin. Exposure time was one hour and mortality was recorded 24 h later. Approximately 100 mosquitoes (four replicates of 25 mosquitoes) were used per test and the average mortality and the binomial confidence interval (95 %) calculated.

WHO susceptibility assays were also used to generate time response data. The standard 0.75 % papers were used but exposure time was varied from 5 min to 20 h (minimum of five time points per strain). The mean mortality was recorded per time point and the LT_{50} estimated using the Dose Effect function on XLSTAT (Microsoft).

CDC bottle bioassays

A modified version of the published Centers for Disease Control and prevention (CDC) bottle bioassays was used to generate dose response data. Glass 250 ml bottles were coated with different concentration of permethrin ranging from 5 μg/ml to 200 μg/ml with between six and nine concentrations used per strain. Bottles were prepared according to CDC guidelines [14], but with a more stringent bottle washing process that involved rinsing them twice with acetone, washing with soap, rinsing with clean water and leaving them overnight in fresh water to eliminate any trace of soap. Approximately 25 mosquitoes were aspirated into the bottles for one hour and subsequently transferred to insecticide free paper cups, with a source of sugar solution, and mortality was recorded 24 h later. Four to six replicates were performed for each concentration with a control bottle (impregnated with acetone only) run alongside each insecticide concentration. Equivalent age mosquitoes from the Kisumu laboratory susceptible strain were exposed to insecticide concentrations ranging from 0.20 μg/ml to 5 μg/ml. The lethal concentration giving 50 % of mortality (LC_{50}) was calculated as above.

Resistance intensity rapid diagnostic test (I-RDT)

This is a simplified version of the CDC bottle bioassay described above in which fixed concentrations of insecticide are used. Four pre-measured vials containing permethrin which, when diluted in acetone and applied to 250 ml bottles give insecticide concentrations 1x, 2x, 5x and 10x (21.5 μg/ml, 43 μg/ml, 107.5 μg/ml and 215 μg/ml, respectively) the diagnostic dose were provided by CDC, Atlanta. These dosages for permethrin are those recommended in the CDC resistance intensity rapid diagnostic test (I-RDT) protocol now included as an insert in the 2010 CDC bottle bioassay manual [14]. Four replicates of 500 μl of acetone were added to each insecticide vial, and then transferred to a falcon tube and a further 48 ml of acetone added. The insecticide solutions were stored at 4 °C in the dark until use. 1 ml of insecticide solution was applied to 250 ml glass bottles and mosquitoes exposed for 30 min as described in the CDC bottle bioassay manual. At the end of the exposure the mosquitoes were transferred to insecticide free paper cups and immediate knockdown was recorded. The mosquitoes were provided with sugar solution, and retained in the paper cups in the insectary for a further 24 h before mortality was recorded.

Cone bioassays

Cone bioassays were performed using Olyset and Permanet 2.0 nets, provided directly by the manufacturer (Sumitomo Chemical Ltd and Vestergaard, respectively). Ten replicates of ten mosquitoes were tested on net pieces selected randomly from the nets. Five replicates were exposed to an untreated net as control experiments. Mosquitoes were exposed for 3 min and the 60 min knock-down and 24 h morality recorded. Significant differences between knockdown or mortality between strains were determined by pairwise comparisons using the z-test and the software programme VassarStats.

Results

WHO susceptibility assays

The percentage mortality after exposure to the WHO discriminating dose of permethrin (0.75 %) for 60 min varied from 100 % for Kisumu to 3.5 % for Tiassalé.

There was no significant difference in mortality rates for Tororo and Tiefora, which both had less than 40 % mortality (Fig. 1).

Exposure time was then varied and the time mortality response plotted (Fig. 2). The time required to obtain 50 % mortality (LT_{50}) was estimated to be 51.5 min (95 % confidence intervals (CIs) 42.5–62.3) and, 97.1 min (95 % CIs 92.0–102.7) for Tororo and Tiefora strains respectively. For Tiassalé the longest exposure time used in the experiment (20 h) only gave 58 % mortality although the best fit curve for the data gave estimated the LT_{50} to be over 22 h. The permethrin LT_{50} for Kisumu females has been previously determined as 7.8 min [15]. Using this Kisumu data as the denominator, the resistance ratios for the three strains according to the LT_{50} values are 6.6-fold for Tororo, 12.4-fold for Tiefora and 174.8-fold for Tiassalé.

CDC bottle bioassays

Keeping a fixed exposure time of 60 min but varying the concentration of insecticides using the bottle bioassays enabled the permethrin concentration required to achieve 50 % mortality to be estimated (Fig. 3). For Tororo this was 12.5 µg/ml (95 % CI 10.9–14.3), for Tiefora 26.5 µg/ml (22.4–31.1) and for Tiassalé 35.8 µg/ml (30.6–40.9). By comparison the LC_{50} for the susceptible Kisumu strain was just 0.23 µg/ml (0.058–0.34) leading to resistance ratios of 54.3-fold, 115.2 -fold and 155.6-fold for Tororo, Tiefora and Tiassalé, respectively.

Resistance Intensity Rapid Diagnostic Test (I-RDT)

An alternative approach to using the CDC bottle bioassays termed the Resistance Intensity Rapid Diagnostic Test (I-RDT) (14) was also evaluated. Pre-measured insecticide vials with different multitudes of the diagnostic dose were used to record the immediate knockdown at the end of the 30 min exposure and the 24 h mortality (Fig. 4). The CDC bottle bioassay guidelines recommend a cut off of less than 90 % knockdown at the end of the assay as the definition of a resistant population. Using this criteria, Kisumu was susceptible to the 1x diagnostic dose (21.5 µg/ml), the Tororo and Tiefora strain were resistant to 1x, but susceptible to the 2x diagnostic dose and Tiassalé was resistant to 1x and 2x but susceptible to 5x the diagnostic dose. However, when 24 h mortality was used at the end point, Kisumu was still susceptible to the 1x dose, but Tiefora was resistant to the 2x dose but susceptible to 5x and both the Tororo and Tiassalé strains were resistant to 5x the diagnostic dose. The highest concentration (215 µg/ml) resulted in almost complete knockdown in all three strains and >90 % mortality.

Cone bioassays

When mosquitoes were exposed to a new Olyset Net LLIN, which has permethrin incorporated into the polyethylene fibres, mortality rates less than 50 % were observed for all strains, including the Kisumu susceptible strain (Fig. 5). Exposure to PermaNet 2.0, whose polyester fibres are coated with deltamethrin, resulted in

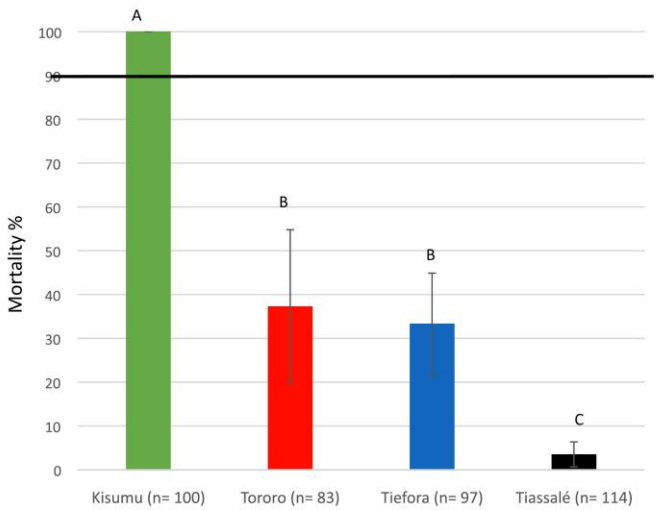
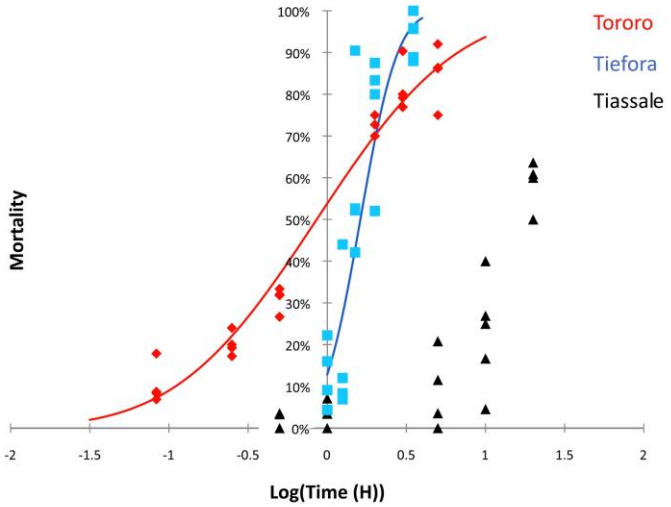


Fig. 1 Discriminating dose. Mortality rates after exposure to the WHO discriminating dose of permethrin. Error bars represent 95 % binomial confidence intervals. Columns with a different letter are significantly different to each other. The 90 % threshold used by WHO to define a resistant population is shown by a horizontal line



	Total Sample Size	LT_{50} (minutes)	Lower bound 95%	Upper bound 95%
Tororo	581	51.5	42.5	62.3
Tiefora	530	97.1	92.0	102.7
Tiassalé	531	1363.6	1035.6	199.2

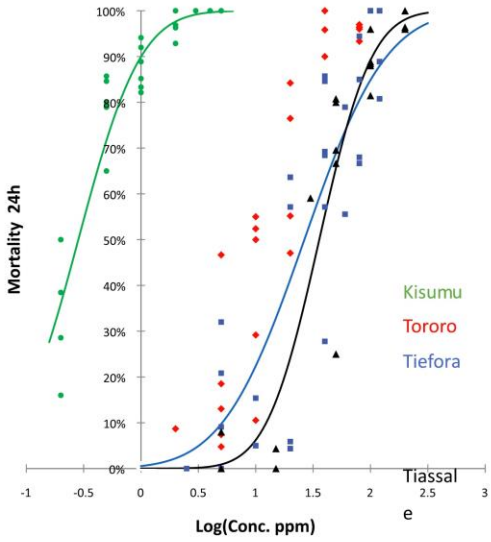
Fig. 2 Time response. Time response curves for adult females exposed to 0.75 % permethrin in WHO susceptibility tube assays. Best fit lines are plotted using XLSTAT. Tiefora and Tororo were exposed for five or six different time points ranging from 5 to 300 min. Tiassalé was exposed four time points from 30 min to 20 h

100 % mortality in the susceptible laboratory strain but with less than 80 % mortality in all three resistant strains (Fig. 5). For both nets mortality rates were lowest for the Tiassalé strain, followed by Tiefora and highest mortality was seen for Tororo.

Discussion

In 2012, the WHO published the Global Plan for Insecticide Resistance Management in malaria vectors (GPIRM) in response to the growing number of countries reporting insecticide resistance in Anopheles mosquitoes [1]. This document provides information on current monitoring guidelines and outlines alternative resistance management strategies that countries can adopt to mitigate, or preferably delay, the onset of insecticide resistance. However, all substitutes to pyrethroids for IRS are more expensive and the only alternative to pyrethroid-treated LLINs currently available are nets containing pyrethroid and PBO, which, again, have a higher unit cost. Therefore, malaria control programmes face challenging decisions when insecticide resistance emerges. A textbook insecticide resistance management strategy requires a change in insecticide class as soon as there is any sign of resistance in a population. But in reality, indications of control failure with insecticide are likely to be the trigger for a change in insecticide use. An indication of the threshold at which resistance is likely to negatively impact on control would aid decision making by providing pragmatic guidance on when it is necessary to respond. The GPIRM document does recommend alternative strategies depending on the resistance mechanism(s) involved, but mechanistic studies are not always feasible in settings with constraints on available expertise and resources. To define this threshold of 'operationally significant resistance', it is necessary to agree on a standardized method for quantifying the strength of resistance that can supplement information on resistance prevalence that is already being routinely generated.

The current study compared results obtained using different bioassay methodologies. Three populations were investigated that were all classified as resistant according to



	Total Sample Size	LC_{50} (µg/ml)	Lower bound 95%	Upper bound 95%
Kisumu	615	0.28	0.23	0.33
Tororo	497	12.5	10.9	14.3
Tiefora	514	26.5	22.4	31.1
Tiassalé	493	35.8	30.6	40.9

Fig. 3 Dose response. Dose response curves for adult females exposed to permethrin for 60 min in CDC bottle bioassays. Best fit lines are plotted using XLSTAT. A minimum of six concentrations were used for each strain

the current WHO definition (Fig. 1) plus a laboratory susceptible strain as a comparator. Two of the resistant populations were a mixture of two *An. gambiae* complex species, *An. gambiae s.s.* and *An. coluzzii*. For the Tiefora population, in which *An. gambiae* was the major vector, the study verified that there was no significant difference in the species composition between the general population and those surviving the 2 x intensity assay ($p = 0.757$). Tiassalé has been maintained in colony for multiple generations and has a large proportion of hybrids. Both single species and hybrids survive the diagnostic dose. The presence of mixed populations could be seen as a weakness of the study but does reflect the reality of many field studies, where species ID is not possible.

The LT_{50} and LC_{50} measurements both ranked the strength of resistance as highest in Tiassalé, followed by Tiefora and lastly Tororo although the confidence intervals for the LC_{50} for Tiefora and Tiassalé overlap. Using the I-RDT and 24 h mortality as an end point, Tiassalé and Tororo both fall into the same category with Tiefora showing a lower level of resistance but when knockdown is used, Tiassalé is categorized as being in a higher resistance class than either Tororo or Tiefora.

The two fully quantitative assays proved challenging at different ends of the resistance spectrum. Reliable measurements for the LT_{50} could be obtained for susceptible strains and populations with relatively low levels of resistance but this methodology was not well suited for the Tiassalé strain as, even at the maximum exposure time (20 h) high levels of mortality were not obtained (Fig. 2). Such high exposure times may result in mortality that is due to factors other than the insecticide itself,

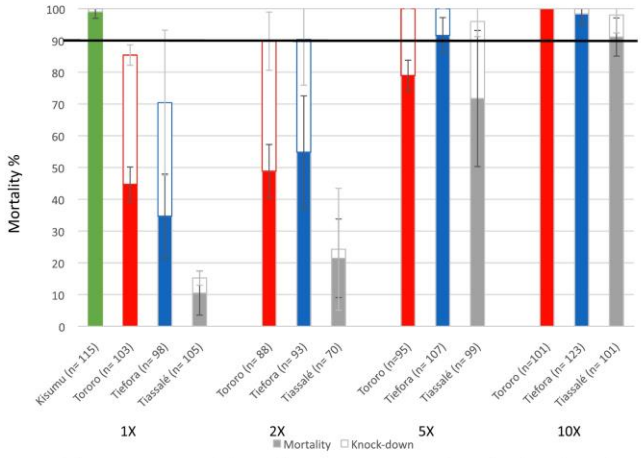


Fig. 4 CDC resistance intensity rapid diagnostic test. Knockdown after a 30 min exposure (open box), and mortality after a 24 h recovery (solid box) to bottles coated with different multitudes of the CDC bottle bioassay diagnostic dose for permethrin. Error bars represent 95 % binomial confidence intervals. The 90 % threshold used by CDC to define a resistant population is shown by a horizontal line

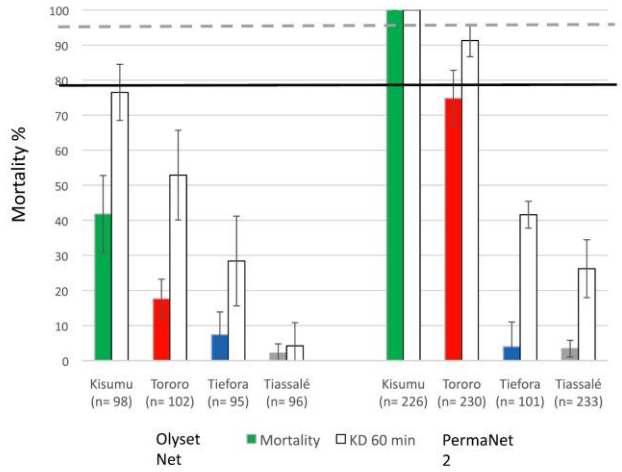


Fig. 5 Cone bioassays. Knockdown after a 3-min exposure (open box), and mortality after a 24-h recovery (solid box) to long-lasting insecticide-treated bed nets using a cone bioassay. Error bars represent 95 % binomial confidence intervals. The solid line and the dotted line represents the 80 % mortality and 95 % knockdown threshold used by WHO to test the efficacy of LLINs in phase I studies

although in this study, the mortality in the control tube after 20 h was zero ($n = 52$). This method was very easy to perform in the field.

For the LC_{50} calculations, difficulties arose at the other end of the spectrum. Here it proved difficult to obtain a reliable value for the susceptible Kisumu strain with the lowest concentration giving 33 % mortality (Fig. 3). The problem is even more acute for deltamethrin where the quantities of insecticide that need to be measured are an order of magnitude lower. Measuring resistance strength using the bottle bioassay method was more challenging to perform under field settings as it required access to technical grade insecticide and a fine scale balance. The stringency of the bottle washing procedure also needed to be increased to avoid cross contamination issues.

Despite these challenges, the permethrin resistance ratio calculated for the Tiassalé strain, using Kisumu as the denominator, was comparable for the two methods (175-fold using the LT_{50}; 156-fold using the LC_{50}). However wider discrepancies were seen for the measurements for the less resistant Tororo and Tiefora strains where LC_{50} RR estimates were 8–9 × higher than LT_{50}). There is very little published data on resistance strength to compare these data sets to and certainly no studies that have compared resistance ratios obtained using LC and LT data. Resistance ratios of 138 and 292 –fold, calculated using LT_{50} measurements, have been reported in field populations of *An. gambiae s.l.* exposed to deltamethrin in Côte d'Ivoire and Uganda, respectively [13, 15], and a recent study in Zambia (recording knockdown rather than mortality) found time to knockdown with deltamethrin was approximately 14-fold higher in a wild population of *An. funestus* than in the laboratory susceptible strain [16]. The highest resistance ratios reported to date are from Burkina Faso where, in 2012, *An. coluzzii* populations were 650 × more resistant to deltamethrin than the Kisumu strain using LT measurements. The following year, resistance ratios >1000 were reported from the same study site using bottle bioassays and comparing LC50s [12].

The interpretation of the intensity assays results was heavily dependent on whether immediate knockdown (KD) at the end of the assay or 24-h mortality was used as the metric. Using KD, the Tororo strain is borderline susceptible (90 % knockdown). Furthermore, all three strains would be classed in a lower resistance category using KD than mortality. Differences in the outcomes from the two metrics likely reflect the role of different resistance mechanisms. For populations where knockdown resistance or *kdr* is the major contributing resistance mechanism, KD rates might be expected to be lower than mortality, as the target site mutation enables mosquitoes to temporarily withstand pyrethroid exposure. Whereas for metabolic mechanisms, or when the insecticide exposure exceeds the protection from KD afforded by the *kdr* allele, mortality rates may be lower than KD as knocked down mosquitoes are able to detoxify the insecticide rapidly enough to recover after exposure is removed. A primary consideration in selecting the end point to record should be the value of the output to decision making in insecticide use: is a mosquito that survives knockdown or a mosquito that is temporarily

intoxicated but later recovers the greatest threat? The answer to this may be dependent on the mode of application of the insecticide with a mortality being of more relevance for measuring the efficacy of IRS applications and both KD and mortality being of value for assessing the insecticidal activity of pyrethroid-treated nets.

Reaching a consensus on how to measure the strength or intensity of resistance is only the first step. Agreement is also needed on what level of resistance has an operational impact. In the agricultural sector, definitions of operationally significant resistance are often related to the field dose of insecticide by dividing the LC_{50} by the field dose [17]. However this is not so straightforward in vector control as formulation issues can have a major impact on the bioavailability of insecticide making the field dose difficult to determine.

Although responses to the field dose were not quantified in this study, cone bioassays were included to compare how the three populations responded to the bioavailable insecticide on the surface of a LLIN. Using PermaNet 2.0, only the susceptible Kisumu strain met the WHO criteria of > 95 % knockdown and > 80 % mortality. Mortality rates with Tiefora and Tiassalé were below 10 % suggesting that the performance of these nets against these populations would be severely compromised. Results from the Olyset nets were difficult to interpret as the mortality and knockdown rates were below WHO criteria even for the susceptible strain. Previous studies have also reported that Olyset nets perform poorly in cone bioassays [18]. However, it is noted that the mortality with the three resistant strains was significantly lower than the susceptible strain, indicating that resistance is also having an impact on efficacy of this net type. Tunnel bioassays [19] have been proposed as a more realistic measure of the performance of the different net types but these are less amenable to routine monitoring programmes where obtaining the necessary equipment and live animals may be problematic.

An alternative approach to defining an operationally significant resistance threshold would be to utilize data from experimental hut studies. In principle data on the performance of nets could be correlated with the strength of resistance. Whilst this could be done using resistance prevalence data from discriminating dose assays, for reasons outlined above, introducing a more quantitative measure of resistance would improve the rigor of this analysis.

Finally, it should be noted that the source of mosquitoes used in the bioassays could have a considerable impact on the data. It is well documented that age, physiological status and larval rearing environment affects the resistance status of adults [20–22]. In this study the bioassays on field populations were all performed on adults raised form larvae to standardize between assays but this is not always practical, nor indeed desirable. It could be argued that the information of most value to control programmes is the resistance level of the entire population of potential vectors which would argue for bioassays directly on indoor (or outdoor) adult collections. As usual, the best approach depends on the question being asked. If comparing response between sites, or over time is the goal then some element of standardization on which mosquitoes are tested is necessary but if a quick assessment of the probably resistance status in a location is what is required, the use of wild caught adults may suffice.

Conclusion

The objective of this study was to compare the results, and practicalities, of using alternative measures to quantify the level of resistance to a single insecticide in different populations. Each method has its own merits and disadvantages and there were notable differences in the results obtained from each bioassay. Of the currently available assays, the intensity assays are perhaps the best compromise between ease of performance and data richness but further validation of these assays, and guidelines on data interpretation is still needed. Given the widespread acceptability of the current discriminating dose assays from WHO, consideration should be given to the centralized production of standardized papers impregnated with a range of insecticide concentrations to enable resistance intensity to be estimated. Finally, the value of measuring resistance intensity is dependent on the ability to extrapolate from this data to predict the performance of insecticide based vector control tools in different resistance settings. In this regard, it is recommended that cone bioassays be used to assess the response of local field mosquitoes to the field dose and formulation of insecticide being used and, where possible, controls using susceptible mosquitoes should also be performed. In addition, attempts to correlate resistance strength in *Anopheles* mosquitoes with epidemiological indicators of malaria should be intensified.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HR designed the study. JB, NG and RC conducted the experimental work and JM, JB and NG analysed the data. WGB developed the CDC assays and SN supervised the field work. HR drafted the paper and all authors contributed to the draft. All authors read and approved the final manuscript.

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of Science, Technology and Innovation (COLCIENCIAS). The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of organizations they represent.

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Pyriproxyfen is metabolized by P450s associated with pyrethroid resistance in *An. gambiae*

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ABSTRACT

Pyrethroid resistance is widespread in the malaria vector *Anopheles gambiae* leading to concerns about the future efficacy of bednets with pyrethroids as the sole active ingredient. The incorporation of pyriproxyfen (PPF), a juvenile hormone analogue, into pyrethroid treated bednets is being trialed in Africa. Pyrethroid resistance is commonly associated with elevated levels of P450 expression including CYPs 6M2, 6P2, 6P3, 6P4, 6P5, 6Z2 and 9J5. Having expressed these P450s in *E. coli* we find all are capable of metabolizing PPF. Inhibition of these P450s by permethrin, deltamethrin and PPF was also examined. Deltamethrin and permethrin were moderate inhibitors (IC_{50} 1−10 μM) of diethoxyfluorescein (DEF) activity for all P450s apart from CYP6Z2 (IC_{50} > 10 μM), while PPF displayed weaker inhibition of all P450s (IC_{50} > 10 μM) except CYP's 6Z2 and 6P2 (IC_{50} 1−10 μM). We found evidence of low levels of cross resistance between PPF and other insecticide classes by comparing the efficacy of PPF in inhibiting metamorphosis and inducing female sterility in an insecticide susceptible strain of *An. gambiae* and a multiple resistant strain from Cote d'Ivoire.

1. Introduction

Malaria control is reliant on the use of insecticides. The dramatic reductions in malaria cases in Africa that have occurred over the last 15 years have been largely attributed to methods targeting the adult mosquito, primarily via the use of long lasting insecticidal nets (LLINs) treated with pyrethroids and, to a lesser extent indoor residual spraying (IRS) with pyrethroids and DDT and, more recently, carbamates and organophosphates (Bhatt et al., 2015; Ranson and Lissenden, 2016). Resistance to pyrethroids is now widespread in the major malaria vectors in Africa with resistance to other classes of public health insecticides also on the increase (Ranson and Lissenden, 2016). There is therefore an urgent need both for new insecticides to maintain the efficacy of these proven tools, and for new tools to reduce malaria transmission by the mosquito.

Pyriproxyfen (PPF) is a juvenile hormone analogue that inhibits metamorphosis. It has been used for several decades to protect against cotton pests (Carriere et al., 2012) and its extremely low toxicity to humans has also enabled applications in public health such as addition to water storage containers to control *Aedes* populations (Darriet and Corbel, 2006; Lee, 2001). As PPF is active in very low concentrations, the active ingredient can be disseminated by the insect itself; this autodissemination route was shown to be effective at controlling *Aedes* populations in trials in Peru (Devine et al., 2009). For malaria vectors, difficulties in identifying and treating the diverse breeding sites for malaria vectors have so far largely confined larviciding for malaria control to easy to reach urban areas but the possibility of using autodissemination strategies to distribute PPF to target *Anopheles* oviposition sites in rural areas is being explored. Currently, however, control of *Anopheles* mosquitoes is more commonly targeted at the adult stage, hence the impact of PPF on embryogenesis, shows the greatest promise for malaria control. Exposure to PPF effectively sterilizes female mosquitoes and has also been shown to reduce adult longevity (Ohashi et al., 2012; Ngufor et al., 2014). Sumitomo Chemicals Ltd has developed a LLIN incorporating both permethrin and PPF. This

Abbreviations: PPF, pyriproxyfen; ALA, 5-Aminolevulinic acid; DEF, diethoxyfluorescein; CPR, cytochrome P450 reductase.

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0–1.6 mM or 0–3.2 mM respectively) to the reaction mix to titer their effect on the insecticide turnover (fixed at 20 μM). Three replicates of positive and negative control reactions were run for each P450/inhibitor combination.

2.5. High-pressure liquid chromatography (HPLC) analysis

Samples were analyzed by high-pressure liquid chromatography, HPLC (Agilent 1100 series). The quantity of insecticide remaining in the samples was determined by reverse-phase HPLC with a monitoring absorbance at 232 nm using a C18 column, Acclaim 120, Thermo Scientific. 100 μl of sample was loaded with a flow-rate 1 ml/min at 23 °C into an isocratic mobile phase 90% methanol and 10% water. The retention time for PPF is 7.4 min and for PBO 7.1 min.

The same conditions were applied for HPLC analysis for the PPF/permethrin inhibition assays. Retention time for trans- and cis-permethrin is 11.8 min and 14.1 min respectively.

2.6. Mass spectrometry analysis for pyriproxyfen metabolism

PPF metabolism by CYP6P3 was examined by mass spectrometry to confirm oxidation and identify the metabolites produced. Aliquots (5 μl) of organic solvent-quenched reaction supernatant were injected onto a high resolution Thermo Q-Exactive mass spectrometer (MS) that was coupled to a 1290 series Agilent LC system. The chromatographic separation was performed on a Waters Acquity BEH C18 (2.1 × 50 mm; 1.7 μm) analytical column at 30 °C using a mixture of water (0.1% formic acid) (A) and acetonitrile (0.1% formic acid) (B) as mobile phase. In the 12 min run time the gradient program was as follows: 5% B in 0–1 min; 5% B to 100% B in 1–8 min; 100% B in 8–10 min; 100% B to 5% B in 10–10.1 min; 5% B in 10.1–12 min. During the analysis the MS was operated in positive ion full scan mode (mass range: 100–1000 m/z) at 35 K resolution using a constant heated electrospray capillary temperature (320 °C), spray voltage (3500 V), sheath gas (55 arbitrary units) and auxiliary gas flow rate (10 arbitrary units).

2.7. Diethoxyfluorescein metabolism

Diethoxyfluorescein (DEF) substrate was dissolved in DMSO, with final concentration of 2% per assay. All test compounds were dissolved in DMSO, with a final solvent concentration <2% per assay. For calculation of the kinetic parameters (K_M and V_{max}), each P450 was used at a final concentration of 10 nM (1 pmol/reaction) and DEF concentrations in the range: 0, 0.31, 0.63, 1.25, 2.5, 5, 10 and 20 μM. DEF reactions were carried out at 25 °C in 50 mM KPi at pH 7.4 containing 1 mM glucose-6-phosphate (G6P), 0.1 mM $NADP^+$, 0.25 mM $MgCl_2$, and cytochrome b_5 at a 10:1 M ratio, b_5:P450. $NADP^+$ and G6P were excluded from the minus NADPH controls.

Variable ligand concentrations were used for IC_{50} calculations with DEF used at ~ K_M for each P450 (i.e. 0.5, 1.4, 0.7, 1.0, 3.5 and 0.5 μM for CYP6M2, CYP6P2, CYP6P3, CYP6P4, CYP9J5, and CYP6Z2 respectively) and 0.1 μM P450. Three replicates of positive and negative control reactions were run for each P450/substrate combination in opaque white 96-well (flat-based) plates in triplicate. The fluorescent reactions were monitored in a fluorescence plate-reader (Ex = 485 nm, Em = 520 nm) continuously over 20 min time period after the addition of NADPH regenerating system. The rate of fluorescent molecules produced per P450 molecule per min (turnover) was determined by linear regression of the measurements between 3 min and 10 min after the reactions began. The Michaelis-Menten and IC_{50} fitting calculations were performed using Graphpad Prism 6. Data were fitted to the dose-response model and plots with $R^2 < 0.95$ were rejected.

2.8. In vivo studies

Two strains of mosquitoes were used to assess the impact of exposure to PPF on life history. The Kisumu strain of *An. gambiae* originates from Kenya and is susceptible to all insecticide classes used in public health whereas the Tiassalé strain from Cote d'Ivoire shows resistance to four classes (pyrethroids, carbamates, organophosphates and the organochlorine DDT) (Edi et al., 2012). Resistance in the Tiassalé strain is mediated by multiple mechanisms, including the overexpression of cytochrome P450s, notably CYP6P3, 6P4 and 6M2 (Edi et al., 2014). Both mosquito strains were reared in the insectaries at the Liverpool School of Tropical Medicine under a 12:12 photoperiod at 27 °C and 70–80% humidity.

To measure the effect of PPF on metamorphosis, SumiLarv®0.5G (Sumitomo Chemicals Ltd) was ground into a fine powder and dissolved in water to prepare a stock solution of 1000 ppm SumiLarv (50 ppm active ingredient). The solution was left overnight dissolving on a magnetic stirrer, protected from light. Serial dilutions were prepared and the following PPF concentrations were tested: 0.001 ppb, 0.005 ppb, 0.07 ppb, 0.1 ppb, 1 ppb, 5 ppb and 10 ppb. Four replicates of 25 3rd instar mosquitoes were exposed to each of the SumiLarv concentrations in paper cups for up to 8 days. Larvae were fed Tetramin® baby fish food every day and cups covered with netting to prevent adults escaping. The number of live and dead larvae, pupae and adults was recorded every 24 h until all individuals were emerged as adults or dead. Adults and dead pupae were removed daily. The Dose Effect function on XLSTAT (Microsoft) was used to estimate the concentration resulting in 50% emergence inhibition (EI50).

To compare the impact of PPF on adult mosquitoes of the two strains, we measured the ability of this compound to impair ovary development. Borosilicate glass tubes (30 cm long, 11 mm wide) were impregnated with three different concentrations of PPF (ai): 0.55 mg/m^2, 2.75 mg/m^2 and 5.5 mg/m^2. An additional tube impregnated only with the solvent (acetone) was used as a negative control. Tubes were used on the day of preparation. Two groups of fifteen 5–7 days old female mosquitoes from Tiassalé and Kisumu strains were tested for each concentration (n = 30). After 60 min acclimation in paper cups, they were transferred to the glass tubes and exposed for 3 min. Mosquitoes were then returned to the paper cups and left for 24 h with a 10% sucrose solution. 24 h after exposure the mosquitoes were bloodfed and any mosquitoes which did not feed were removed. Mosquitoes were retained in insectary conditions with access to sugar water for five days and then dissected and the morphology of the ovaries assessed as normal (loose, well developed eggs) or abnormal (non-detachable, bubble-like eggs). Dead mosquitoes or mosquitoes not presenting egg development were discarded and removed from the analysis.

To confirm that formulated products containing PPF also impaired egg development, mosquitoes were exposed to Olyset Duo nets, nets containing 1% PPF only (supplied by Sumitomo Chemicals Ltd), conventional Olyset nets or untreated nets. Batches of 10 3–5 days old Tiassalé mosquitoes were exposed to the nets for 3 min following WHO standard protocols for cone bioassays. Twenty-four hours after the exposure, mosquitoes were bloodfed and left in paper cups with 10% sucrose solution. Dead and non-bloodfed mosquitoes were removed from the experiment. After 5 days surviving mosquitoes were dissected and the morphology of the ovaries examined as described above. To assess the effects of short exposures to PPF LLINs, an additional set of cone bioassays was performed, as described above, except exposure time was reduced to 30 s.

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3. Results and discussion

This study was motivated by concerns over cross resistance between pyrethroid insecticides and the insect growth regulator, PPF. Cross resistance between insecticide classes with different modes of action, mediated by cytochrome P450s, has previously been demonstrated in *An. gambiae* (Edi et al., 2014; Mitchell et al., 2012) and here we investigated whether these same P450 enzymes can also metabolize PPF. CYP6M2, CYP6P3 and CYP6Z2 have previously been expressed in bacterial expression systems but we extended the panel of recombinant enzymes to include a further three *An. gambiae* CYP6P P450s implicated in pyrethroid resistance in Cote d'Ivoire (Edi et al., 2014) and CYP9J5, which has been found over expressed in pyrethroid resistant populations from Bioko Island (Hemingway et al., 2013) and Burkina Faso (Toé et al., 2015).

3.1. Functional expression of P450s in E. coli

P450s require electrons from NADPH-cytochrome P450 oxidoreductase (CPR) for catalysis, thus new candidate *An. gambiae* P450s were co-expressed with AgCPR in *E. coli* using ompA and pelB leader sequences to direct the enzymes to the inner bacterial membrane as previously with CYP6M2 and CYP6P3 (Muller et al., 2008; Stevenson et al., 2011). CYP's 6P1, 6P2, 6P4, 6P5, 9J5 and 6M1 were co-transformed with AgCPR-pACYC for *E. coli* expression, producing characteristic CO-reduced spectra indicative of active P450 (Fig. S1). The yields of P450 were in the range 10–100 nmol/l, with CYP6P5 producing the lowest quantities of P450 (~10 nmol/L; Table S3). CYPs 6P1 and 6M1 failed to express functional P450.

CYP6M2 and CYP6P3 have previously been expressed following co-transformation with AgCPR on separate plasmids. Here, these enzymes were co-expressed in tandem with AgCPR on a single from the P450 expression plasmid, pCWori + to facilitate scaled 10 l fermentor expression. Tandem expression from the single plasmid produced higher CPR:P450 ratios. The fluorogenic substrate DEF was used to estimate the kinetic parameters of the tandemly expressed recombinant proteins against dual plasmid protein expression (Table 1) and used in dose-response experiments to determinate the inhibitory effect of permethrin, deltamethrin and pyriproxyfen in its metabolism. As expected, since CPR is rate limiting, the V_{max} values were 3–6 fold higher using the single plasmid in tandemly expressed membranes compared with dual plasmid expression, consistent with the elevated levels of CPR. The K_M value is the concentration of DEF required to reach max reaction velocity and independent of the enzyme concentration. The single versus double plasmid K_M values for CYP6M2 (0.4 vs 0.5 μM) and CYP6P3 (0.7 vs 0.9 μM) were similar, again consistent with higher CPR levels increasing reaction rates through enhanced electron transfer rather than effects on substrate binding.

3.2. PPF metabolism

The ability of the *An. gambiae* P450s to metabolize PPF was tested by measuring substrate turnover (substrate disappearance over time) in the presence and absence of NADPH. We also included PBO in parallel reactions as further validation of P450 induced substrate depletion (Fig. S2). PBO is an inhibitor of P450 monoxygenase activity and a common insecticide synergist (Vijayan et al., 2007). All seven P450s metabolized PPF to some degree with the percentage PPF depletion ranging from 24.78% for CYP9J5 to 100% for CYP6P3, and PPF depletion was inhibited by PBO for each P450 tested (Table 2). Since *E. coli* membranes expressing CYP6M2 and CYP6P3 had higher levels of CPR, the rates of activity were not comparable with the rest of the P450s. However, it is notable that, with the exception of CYP6P5, all members of the CYP6P family and CYP6Z2 produced high levels of PPF depletion (58–100%). CYP6Z2 is of interest since it is found overexpressed in pyrethroid resistant populations of *An. gambiae*, but metabolises the pyrethroid metabolites 3-phenoxbenzoic alcohol and aldehyde rather than the parent compound (Chandor-Proust et al., 2013). Here CYP6Z2 appears to play a direct role in the primary metabolism of PPF, thus may have an influential role in PPF clearance and potentially insecticide resistance.

Mass spectrometry analysis of the PPF metabolites generated by CYP6P3, the strongest metabolizer was carried out to confirm oxidation and identify possible metabolites. Expected metabolites included 4′-OH-PPF, 5″-OH- PPF and 5″,4′-OH-PPF, that have been previously identified from the *in vitro* metabolism of PPF by microsomes from housefly larvae (Zhang et al., 1998). CYP6P3 generated three metabolite peaks (Fig. S3). The extracted ion chromatograms of $[M+H]^+$ generated two peaks (6.9 min and 7.1 min), with molecular mass (m/z = 338.1387) corresponding to the addition of a hydroxyl group (m/z = 16), consistent with 4′-OH-pyriproxyfen and 5″-OH-pyriproxyfen production. Furthermore, the data showed signs of a metabolite with molecular mass 32 m/z larger than PPF, equivalent to a double hydroxylation, potentially 5″,4′-OH-PPF resulting from secondary metabolism of 5″-OH-PPF and/or 4′-OH-PPF metabolites. This was, however, not confirmed chromatographically as analytical reference standards were not available for the analysis. Further collision mass spectrometry or NMR is required to confirm the identity of the metabolites.

Since PPF is being used in combination with pyrethroids in bednets we were interested in potential synergistic effects. We therefore measured the IC_{50} values of PPF, deltamethrin and permethrin to compare relative strengths of DEF metabolism inhibition against the P450s (Table 3). The fluorescent substrate, DEF, was used for monitoring P450 activity using a 96 well microtiter plate format. In drug screens, compounds are generally categorized according to their activity as P450 inhibitors as potent inhibitors (IC_{50} < 1 μM), moderate (IC_{50} 1–10 μM) and weak inhibitors

Table 1
Kinetic parameters for DEF.

P450	Kinetic parameters for DEF			
	Single plasmid expression		Dual plasmid expression	
	K_M (μM)	V_{max} (RFU/sec)	K_M (μM)	V_{max} (RFU/sec)
CYP6M2	0.4 ± 0.02	902.2 ± 106.99	0.5 ± 0.01	292.5 ± 38.05
CYP6P3	0.7 ± 0.10	54.4 ± 6.52	0.9 ± 0.09	14.8 ± 4.89
CYP6P2	nt	nt	1.4 ± 0.02	300.6 ± 12.00
CYP6P4	nt	nt	1.0 ± 0.04	24.2 ± 1.26
CYP9J5	nt	nt	3.4 ± 0.21	265.1 ± 28.75
CYP6Z2	nt	nt	0.5 ± 0.3	43.7 ± 8.9

nt = not tested; (mean ± SD).

Table 2
Pyriproxyfen metabolism by mosquito P450s.

P450	% PPF depletion		Inhibition ratio (%)[a]
	−PBO	+ PBO	
CYP6M2	30.93 ± 4.65	1.44 ± 0.67	95.3
CYP6P2	58.03 ± 1.35	6.45 ± 3.22	91.0
CYP6P3	100.0 ± 0.01	8.68 ± 1.74	91.3
CYP6P4	81.63 ± 0.63	4.44 ± 1.99	94.6
CYP6P5	39.96 ± 1.04	2.40 ± 2.01	95.4
CYP9J5	24.78 ± 2.13	0.08 ± 0.15	99.6
CYP6Z2	66.26 ± 3.02	8.41 ± 1.58	87.3

[a] Reduction of PPF depletion in percentage caused by the inhibitory effect of PBO; (mean ± SD).

Table 3
IC_{50} values for mosquito P450s.

P450	IC_{50} (μM)		
	Pyriproxyfen	Deltamethrin	Permethrin
CYP6M2	14.14	4.24	8.07
CYP6Z2	2.23	13.99	13.72
CYP6P2	9.95	4.97	8.61
CYP6P3	15.82	3.17	6.77
CYP9J5	18.96	6.05	6.47

(IC_{50} > 10 μM) (Krippendorff et al., 2007). Using these criteria, PPF displayed moderate inhibition of DEF metabolism for CYP's 6Z2 and 6P2, with the remainder being weakly inhibited. Deltamethrin and permethrin were moderate inhibitors of DEF activity for all P450s apart from CYP6Z2, which was weakly inhibited by both pyrethroids. As Olyset Duo nets contain both PPF and permethrin, we also measured PPF inhibition of permethrin metabolism by CYP6P3 and vice versa (Fig. 1). CYP6P3 was chosen as it is one of the P450s most frequently found at elevated levels of expression in pyrethroid resistant populations of *An. gambiae*. Permethrin produced slightly stronger inhibition of PPF metabolism (IC_{50} = 61.2 μM) than PPF inhibition of permethrin metabolism (IC_{50} = 92.7 μM). Overall, the *in vitro* results suggest that the pyrethroids deltamethrin and permethrin are slightly stronger inhibitors (2–3 fold) than PPF against the pyrethroid metabolizing P450s tested.

These data indicate that PPF can be metabolized by a wide range of P450s associated with pyrethroid resistance. In mosquitoes that have elevated levels of expression of one or more of these enzymes, it is feasible that enhanced metabolism of PPF could reduce the efficacy of this juvenile hormone analogue. To test this hypothesis we performed PPF bioassays on insecticide susceptible and resistant strains.

3.3. Bioefficacy of PPF against insecticide resistant mosquitoes

The impact of exposure to PPF on metamorphosis and embryogenesis was compared in the insecticide susceptible Kisumu strain and the multi resistant Tiassalé strain from Cote d'Ivoire. The dose-response tests showed that SumiLarv® 0.5G affected adult mosquito emergence in both strains but the minimum dose that inhibited 100% emergence was 1 ppb in the insecticide susceptible Kisumu strain and 10 ppb in the Tiassalé strain. The concentration that resulted in 50% inhibition of emergence (EI_{50}) for the susceptible Kisumu strain was 0.088 ppb (95% confidence intervals 0.064–0.123 ppb) (Fig. 2), similar to values reported for *An. gambiae s.l.* in other studies (0.025 ppb Kawada (1993), 0.13 ppb (Mbare et al., 2013)). In contrast, the EI_{50} for the Tiassalé strain was 0.356 ppb ai (0.274–0.463) approximately 4-fold higher than the Kisumu strain. It is important to note that we cannot directly link the higher EI50 for PPF to the presence of elevated P450s in the Tiassale strain given that only one pyrethroid resistant population was evaluated and it is not known whether the same P450s found elevated in adults of this strain are also up-regulated at the larval stage. Furthermore, the field dose of SumiLarv 0.5G ranges from 10 to 50 ppb so it is likely that the product would still inhibit development of the Tiassalé strain under field conditions. However, given the trajectory of increasing pyrethroid resistance in both *Anopheles* (Ranson and Lissenden, 2016) and

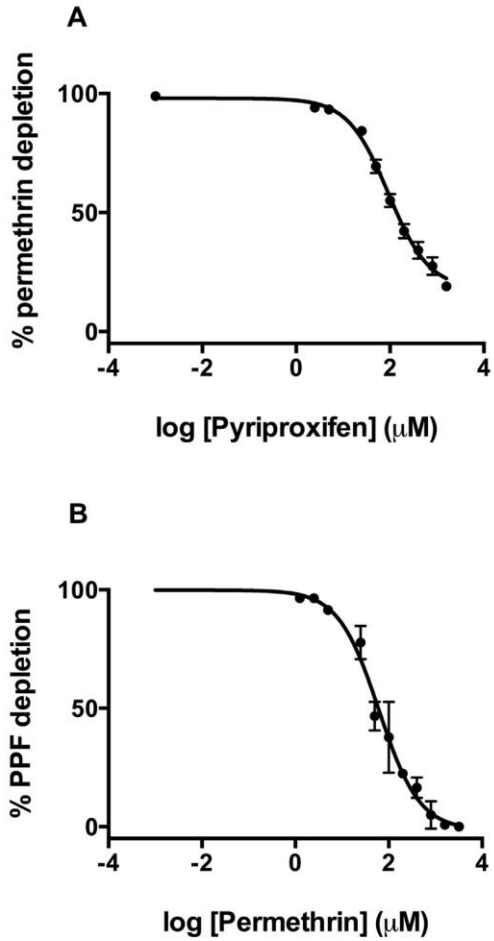

Fig. 1. Determination of IC_{50} values of permethrin and PPF in P450 metabolism. Dose-response analysis of the inhibitory effect of (A) pyriproxyfen on permethrin metabolism and (B) permethrin on pyriproxyfen metabolism.

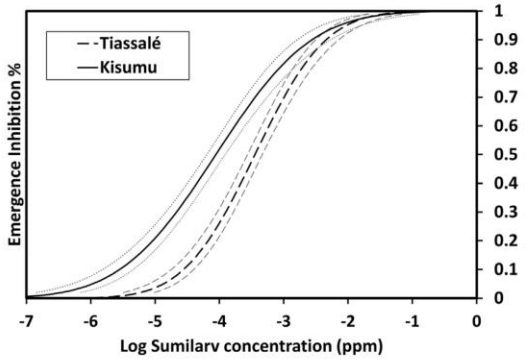

Fig. 2. SumiLarv emergence inhibition curves for two strains of *An. gambiae*. Emergence inhibition dose response curves for the insecticide susceptible Kisumu strain (continuous line) and insecticide resistant Tiassalé strain (dotted line). The grey dotted lines represent 95% upper and lower limits.

Aedes mosquitoes (Bariami et al., 2012) and the growing interest in use of PPF to target immature populations of these vectors (Kiware et al., 2015; Abad-Franch et al., 2015) it is important that further evaluation of PPF efficacy against field populations is carried out.

As the sterilizing effect of PPF on mosquito populations has also received considerable attention for malaria control, with clinical trials of the PPF/permethrin Olyset Duo LLIN ongoing (Tiono et al., 2015; Sagnon et al., 2015) we also compared inhibition of embryogenesis in our susceptible and resistant populations. In a narrow bore glass tube assay, designed to ensure complete contact with the PPF for the duration of the assay, mosquitoes from the insecticide susceptible Kisumu strain were completely sterilized after a 3 min exposure to 5.5 mg/m^2 whereas only 75% of Tiassalé mosquitoes were sterilized by this dose. At half this dose, PPF had no impact on ovary development in Tiassalé but resulted in 76% of Kisumu mosquitoes being sterilized (Fig. 3). Thus higher concentrations of PPF are needed to sterilize the pyrethroid resistant strain, consistent with the effect on metamorphosis described above.

3.4. Interactions between PPF and permethrin

The *in vitro* analysis demonstrated that the same P450s can bind and metabolize PPF and pyrethroids. This interaction could have a synergizing effect with one chemical essentially reducing the rate of depletion of the other such that both chemicals are more potent when used in combination. As the pyrethroid IC_{50}s were generally lower than for PPF (with the exception of CYP6Z2), pyrethroids might be expected to have a stronger enhancing effect on PPF activity than vice versa. However, the differences in IC_{50} values were small (~2 fold) and, as the *in vivo* concentrations in mosquitoes after exposure to products containing PPF and/or permethrin are unknown, such predictions in isolation are highly speculative. Furthermore, as shown above, the performance of PPF varies between strains and thus the impact of combining the two chemistries in vector control products may depend on the level of expression of P450s in the strain.

To investigate this further we evaluated the performance of LLINs containing a single active ingredient versus the combination Olyset Duo LLIN in cone bioassays. The Tiassalé strain was exposed to four net types, mortality recorded 24 h after exposure and surviving mosquitoes were offered a bloodmeal. Ovary development was assessed after a further 5 days. As expected very low mortality

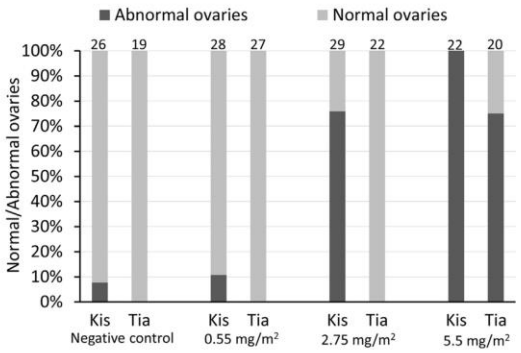

Fig. 3. Effect of pyriproxyfen on egg development for two strains of *An. gambiae*. Proportions of normal/abnormal ovaries of mosquitoes from Tiassale and Kisumu strains exposed to three different concentrations of pyriproxyfen. The number over each bar corresponds to the sample size for each treatment.

Table 4
Impact of exposure to LLINs containing permethrin and/or pyripoxyfen on mosquito mortality and egg development. *An. gambiae* Tiassalé strain were exposed to the LLINs for 3 min. Mortality was measured 24 h later and surviving mosquitoes offered a blood meal. Ovary dissections were performed 5 days later. n bloodfed accounts for the number of surviving mosquitoes that fed on blood and survived for five days until ovary dissections.

LLIN	n	Mortality % (95%CI)	n bloodfed	Abnormal	% Sterilized
Untreated	45	2.22 (0.12–13.2)	23	0	0
Olyset	46	30.4 (18.2–45.9)	19	0	0
Olyset Duo	46	54.3 (39.2–68.8)	10	6	60
PPF	46	2.17 (0.11–13.0)	35	35	100

was observed in the mosquitoes exposed to untreated or PPF only nets (Table 4). Mortality was higher after exposure to Olyset Duo nets than conventional Olyset nets (2-tailed z test, $p = 0.02$). All bloodfed mosquitoes exposed to the Olyset or untreated nets developed normal ovaries. In contrast all of the mosquitoes exposed to the 1% PPF net were sterilized. The number of surviving mosquitoes that successfully bloodfed from the Olyset Duo arm was small but surprisingly only 60% of these mosquitoes were sterilized. The differential sterilizing effect of nets containing 1% w/w PPF alone and Olyset Duo (with 1% PPF and 2% permethrin) was confirmed in follow up cone bioassay study in which mosquitoes were only exposed for 30 s. Here >87% of Tiassalé mosquitoes exposed to PPF nets (n = 34 dissections) were sterilized versus 0% for untreated nets (n = 39) and only 16% for Olyset Duo nets (n = 38) (Table S4).

Taken together these results suggest that PPF *increases* the efficacy of permethrin but permethrin *reduces* the efficacy of PPF. This is supported by data showing higher mortality rates in huts with Olyset Duo than with Olyset in areas with resistant mosquitoes (Ngufor et al., 2014 in Benin, Koffi et al., 2015 in Cote d'Ivoire) (although no increase in the proportion of mosquitoes sterilized in huts containing PPF only nets versus Olyset Duo nets was observed, as our laboratory data would have predicted). It is important to note that these experiments are conducted on formulated products, and although the concentration of permethrin and PPF does not differ between net types, they may differ in their bleed rates affecting the bioavailability of the two active ingredients. Further laboratory and field evaluations, against vectors with differing levels of metabolic resistance, are needed to better predict the performance of combination products, such as Olyset Duo, in the field.

4. Conclusions

Given that pyrethroid resistant populations of malaria vectors are now ubiquitous in Africa, it is important to evaluate the possible impact of this resistance on the performance of any new vector control tools. In this study we demonstrated that a subset of mosquito P450 enzymes responsible for elevated pyrethroid metabolism in insecticide resistant mosquitoes can also metabolize PPF. As metabolic resistance is an increasingly problematic resistance mechanism in African malaria vectors, there is a very real concern that PPF resistance may already be present in field population of *Anopheles* mosquitoes. Although the levels of PPF resistance we observed in the current study are low, continual monitoring for resistance to this chemistry should be undertaken in any area employing PPF as a larvicide or considering PPF use for adult mosquito control.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2016.09.001.

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